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SUPPLEMENTA

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DEPARTMENT OF ORTHOPAEDIC SURGERY UNIVERSITY OF MALAYA
SINGAPORE

OSTEOGENIC SARCOMATOSIS OR MULTIFOCAL OSTEOGENIC SARCOMA

By

A. Karlén

Received 9 xi 61

In the last edition (1959) of *Lichtenstein*s Bone Tumours the spread of an osteogenic sarcoma is described as follows "Metastasis in osteogenic sarcoma occurs almost exclusively by haematogeneous spread and extension to regional lymphnodes although it has been noted to appear to be of infrequent occurrance Pulmonary metastases are consistently present in cases which come to autopsy On occasion one may observe late metastases in one or more of the other bones but visceral metastases aside from those in the lungs are unusual

With intervals cases have been reported in the literature under the name of "Multifocal or Multicentric Osteogenic Sarcoma Sclerosing Osteogenic Sarcomatosis Osteogenic Sarcoma with Multiple Skeletal Tumours etc which have been interpreted as a separate entity differing from the ordinary osteogenic sarcoma in the respect that the various skeletal tumours were not secondary but primary lesions It is well known that multiple sarcomata arise in Paget s disease and an explanation of the multicentric osteogenic sarcoma has been sought in a presumed inherent abnormal bone growth (*Silverman* 1936 *Moseley & Bass* 1956)

Hitherto it has not been possible to establish the pathological differentiation between a primary osteogenic sarcoma and its skeletal metastases and consequently there is no basis on which to distinguish between a primary osteogenic sarcoma with metastases and lesions of multifocal origin (*Silverman* 1936 *Dresser & Dumas* 1930) The fact that osteogenic sarcomata only rarely metastasize to bone can hardly be used in support of a theory of multicentric origin nor can a differential diagnosis between a primary lesion with metastases and one of multicentric origin be based merely on a time factor

Recently one case was seen which initially was considered to be one of sclerosing multifocal osteogenic sarcoma

I would like to thank Dr M S Muir Department of Pathology University of Malaya for his kind help



Fig 1 Fig 2

Fig 1 —X-ray of both knee joints The right shows a sclerosing sarcoma involving lower end of femur and upper end of tibia In both heads of fibula small dense areas more pronounced on the left side

Fig 2 —X ray of upper end of femora and part of pelvis showing same deposits in pelvis, upper part of both shafts of femur, trochanteric region left and inguinal glands on the right side

CASE REPORT

A 17 year old Indian male First seen on 15 6 1959 Within the last 6 months a gradually increasing swelling of the right knee For the last two months pains in the knee, inability to bend the knee fully and to walk quickly No other complaints 1 or 1 month a swelling in the right groin had been present

Clinical examination Healthy looking boy Chest clinically nil Blood pressure *120/60 mm Hg* Abdomen nil C N S nil

Right knee Swelling 2" × 2" at the medial upper part of tibia Surface smooth Attached to bone but not to skin or soft tissues Consistency bony hard

Right inguinal glands Two hard masses palpable irregular surface

Blood Hb 96%, W B C = Neutr 67 Lymph 23 Eos 8 Mono 2 B S R 5 mm/1 *hour Acid Phosphatase less than 4 units Alkaline Phosphatase 8 units Serum* Albumen = 4 69 gms % Serum Globulin = 3 88 gms % Serum Total Protein = 8 57 gms %

Urine nil

X Ray examination of the right knee Pronounced and widespread bone changes *Ill defined irregular patches of increased density are present in the lower third of* the femur and the upper half of the tibia Except for the medial angle of the eroded tibial table the articular margins of the knee appear free from involvement The cortical bone of the upper tibial extremity is destroyed showing irregular new bone spiculation with periosteal reaction cuffs The cortex on the posterior aspect of the lower femur is also damaged Small faint floccular deposits are noted in the neighbourhood Irregular calcium deposition is seen in the popliteal fossa suggesting involvement of the popliteal gland (Fig 1)

Pelvis Similar dense fluffy opacities are seen in the pelvis notably in the vicinities of the sacro iliac articulations and the acetabular fossae An area of density is also observed in the soft tissues of the right sciatic notch A dense shadow is seen in the right groin indicative of calcium deposit in the inguinal glands or calcified implants (Fig 2)

Chest The right root shadow is enlarged Multiple dense opacities are scattered in both lung fields especially in the lower zones consistent with malignant metastases

Skull A certain hyperostosis of the cranial sutures is seen No radiological evidence of a raised intracranial tension



Fig 3

X ray lumbar spine Small deposit in the antero inferior angle of the body of L 3

Spine There is no gross lesion apart from a deposit in the antero inferior angle of the body of L 3 (Fig 3)

Upper limbs N A D

Lower limbs Fluffy deposits are present in the upper femurs and the left upper fibular extremity (Figs 1 and 2)

Biopsy of right inguinal gland The picture is consistent with a sclerosing osteogenic sarcoma

Sternal puncture Malignant cells are present

1 7 Difficulty in swallowing Tongue deviation caused by a mass at the back Voice slightly hoarse Barium meal Screening and films show no obstruction of the flow of barium into the stomach Treatment with T E M (triethylmelamine) was instituted on July 7 using 5 mg once weekly up to 20 mg The patient is gradually sinking and feeding through tube is required A new lump is appearing on the fore head and occiput an intractable headache prevails The patient died on August 27 about 10 weeks after admission

Autopsy The right knee is moderately swollen the swelling extending up the shaft of the f
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heart is enlar
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and hard as s
in particular
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Pharynx a
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Fig 1 *Fig 2*

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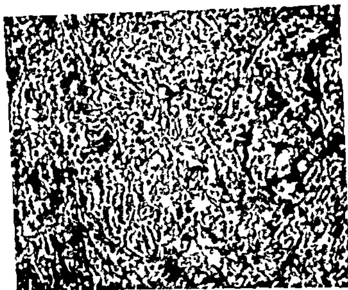
Urine nil

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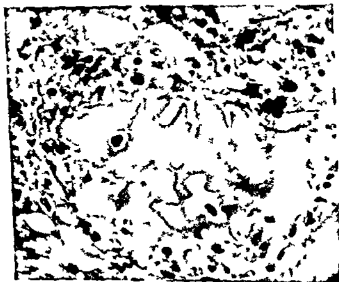
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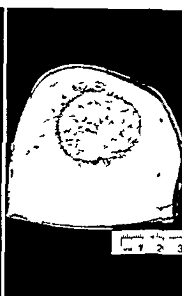


F g 7
Lung secondary Mode ate am unts of calc f e l o te id between sarcomat us cells
H & E × 45



F g 8
Sect n f sternum show ng o te bla tic tum ur in the marrow
H & F × 500





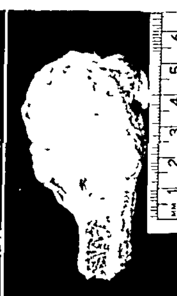


Fig 4 *Fig 5* *Fig 6*

Fig 4 —? primary lesion in upper end of tibia and lower end of femur
Fig 5 —Tumour on the inner table of the skull
Fig 6 —Tumour in manubrium Sections taken from sternum showed involvement

The stomach appears normal but the pancreatic duodenal glands are grossly involved The para aortic and inguinal glands show gross metastases individual glands being up to 5 cm across Both adrenals show a small solitary bony metastasis The spleen is enlarged soft and flabby Both kidneys appear normal The bladder contains 1½ litres of urine and is grossly distended There is no outflow *obstruction of the bladder* The liver is enlarged weighing *1520* grams *It is tense* firm and congested Beneath the capsule are two small linear bony metastases The pancreas is completely replaced by tumour nodules and well calcified No intra cerebral metastases are seen

Microscopically The primary tumour is of the sclerotic type

Large sheets of well formed practically acellular bony tissues merge imperceptibly with areas showing partial calcification of the metachromatic matrix containing moderate numbers of tumour cells These cells have an indefinite eosinophil cytoplasm and large vesicular nuclei The nuclear membrane is sharply defined the sparse chromatin content is condensed into one or two small clumps In such places a few small sinusoidal blood channels lined by a single endothelial layer are present No tumour giant cells are seen

At the periphery of the tumour are focal areas of a highly cellular appearance Although substantially of the same nature as these seen deeper in the tumour the cells are much more pleomophic showing considerable variation in size and in intensity of nuclear staining Binucleate forms and bizarre mitoses are seen a few giant cells are present These peripheral portions of the tumour are much more vascular and where there is invasion of skeletal muscle these endothelial lined blood channels are quite prominent Sclerosing Osteogenic Sarcoma (Figs 7 and 8)

The case called for a careful study of the literature The result is shown in Table 1 The following cases have been excluded *Willis* (1934) not being a case of osteogenic sarcoma *Dresser & Dumas* (1930), first diagnosed as an osteochondroma, on relapse as an osteogenic sarcoma the cases of *Kraft, Peck & Drompp* mentioned by *Lichtenstein* (1959) details not sufficient, *Moseley & Bass* (1956) no autopsy data, and *Busso & Schajowicz* (1945 46) no autopsy data

4 The possibility of an osteogenic sarcoma in adults having a multicentric origin based upon some form of generalized bone dystrophy is discussed

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1 *Ackerman A J* Multiple Osteogenic Sarcoma American Journal of Roentgenology & Radium Therapy *60* 623 1943
2 *Busso M C & Scajowic F* Sarcoma Osteogenica A localization Multiple Revista De Ortopedia & Traumatologie XI 85 1945
3 *Dresser R & Dumas C* An Unusual Case of Osteogenic Sarcoma American Journal of Roentgenology 23 65 1930
4 *Durham A F* Enormous Osteo Sarcoma of the Left Femur In A Boy Trans Path Soc London 34 265 1883
5 *Finlaysen R* [illegible] Path [illegible]
6 *Halpert B* [illegible] Skeleta [illegible]
7 *Lichtenstein* [illegible]
8 *Moseley J F* [illegible] Entity
9 *Price C H C & Truscoth D F* Multifocal Osteogenic Sarcoma The Journal of Bone and Joint Surgery *39B* 524 1957
10 S[illegible]rman C Multiple Osteogenic Sarcoma Archives of Pathology 21 88 1936
11 *Willis R A* The Spread of Tumours in the Human Body London 325 1934

Most likely the latter case may have metastasized to the lungs as well as to the liver which is also pointed out by the authors

These cases are seen to fall naturally into two groups one group of young individuals (cases 1, 2, 3, 4, 5, 6, 8, 9), ages ranging from 7–27 years (average 14 7), and one group of older individuals (case 7) comprising only one case at the age of 50 years

TABLE 1

	Author	Chest metastases	Number of cases	Age	Sex
1	*Durham* (1883)	+	1	9	Male
2	*Silverman* (1936)	+	1	27	Male
3	*Ackerman* (1943)	+	1	10	Male
4	*Ackerman* (1943)	+	1	21	Male
5	*Halpert* (1949)	+	1	7	Male
6	*Finlayson* (1953)	+	1	12	Female
7	*Price & Truscott* (1957)	—	1	50	Female
8	*LeMonchel* (Lichtenstein 1959)	+	1	15	Male
9	Karlén	+	1	17	Male

In the first group, all cases showed, besides the skeletal metastases, also tumours in the lungs, other viscera and lymph glands, probably because the origin in this group is a highly malignant, primary osteogenic sarcoma with a tendency to very early spread In the case belonging to the second group, the generalized osteoporosis was far more pronounced than might be expected in patients at this age, and might be ascribable to some kind of endocrinological dysfunction The microscopic examination showed a low-grade sarcoma with extremely scanty mitosis Hence this case may be brought into alignment with the multiple osteogenic sarcomas known from Paget's disease *i e* caused by a generalized bone disturbance

SUMMARY

(1) *A case of sclerosing osteogenic sarcoma in a 17 year old Indian* male is presented

(2) The occurrence of multiple metastases in viscera and lymph nodes in conjunction with multiple skeletal sarcomatous deposits seems to support the theory that the multicentric sclerosing sarcomatosis is an unnusual form of primary osteogenic sarcoma with high malignancy and a tendency to widespread, early dissemination in the skeleton and viscera

3 The case is extraordinary not only as regards the multiple bony lesions which seem to have started more or less at the same time (femora, right tibia, left fibula, pelvis sacrum, vertebra skull and manubrium sterni) but also in showing the same tumour masses involving lymph nodes in the groins, abdomen and chest and visceral involvement of lungs, pericardium, pancreas, liver and adrenals

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Fig 1

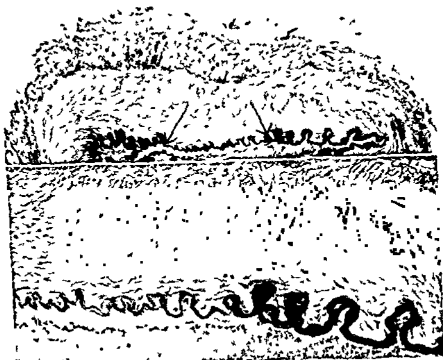


Fig 2

Fig 1 Large area (between the arrows) of weakly staining elastic lamella from case 1 An extra external elastic layer and an increased number of round cells in the adventitia can be distinguished Gomori s aldehyde fuchsin and van Gieson × 40

Fig 2 Higher magnification of the changed elastic lamella (to the left) in Fig 1 × 120

Case 1

eurysm of right
r middle cerebral
a Weight of heart
— 460 g Slight atheromatous changes of the large cerebral arteries

Microscopical findings Three areas with a changed elastic lamella were found in this case One of these was situated in the left anterior cerebral artery about one

amella ranged between 0 05 and 0 2 mm²

The embedded and sectioned elastic lamella was stained again and took up elastin stains fre

imal fenestration

The intima over the changed elastic lamella showed no remarkable changes The

FROM THE HISTOLOGICAL DEPARTMENT (HEAD PROF. M. WRETE), UNIVERSITY OF UPPSALA, SWEDEN

AREAS OF WEAKLY STAINING ELASTIC LAMELLA IN THE CEREBRAL ARTERIES OF PATIENTS WITH INTRACRANIAL ANEURYSMS

By

OVE HASSLER and BJÖRN KIHL

Received 17. viii. 61

The cerebral arteries have an exceptionally thick internal elastic lamina which forms one of the two principal layers of the arterial wall. Almost all of the elastic tissue in the artery is concentrated in this peculiar lamella which is assumed to have important functions. The lamella probably damps the transmission of the pulse-beats to the brain parenchyma (*Ask-Upmark* 1944, *Wolff* 1948). The tendons of the smooth muscle cells of the media insert in the lamella, which is very resistant towards pressure. *Glynn* (1940) found that a free-prepared elastic lamella could withstand pressures up to 600 mm. Hg. At the sites of defects in the media, which are quite common, the elastic lamella probably plays the main role in maintaining the integrity and shape of the cerebral arteries. However, the extremely thick, compact lamella is probably more susceptible to injury and degeneration than the thin internal elastic laminas of other arteries (*Reuterwall* 1923, *Glynn* 1940).

MATERIAL AND METHODS

The material and methods were the same here as in an earlier investigation (*Hassler* 1961). The material included the large cerebral arteries from 250 normal and 11 subarachnoid haemorrhage patients. After fixation by injection of formaline the arteries were stained in toto in a dilute solution of *Gomori's* (1950) aldehyde fuchsin which gave the elastic lamina a deep violet colour. The preparations were placed in glycerine and inspected under a stereomicroscope. Illumination came from a slit lamp which produced a movable optical section through the arterial wall. When a weakly staining area of the elastic lamina was observed it was cut out together with the adjacent 1 cm of the artery situated proximally and distally to it. The preparation was embedded in paraffin and serially sectioned.

RESULTS

In three cases a total of five areas of the cerebral arterial wall were observed which had a more weakly staining elastic lamina than the rest of the wall.



Fig 4

Case 3 The internal elastic lamella is thinner and more weakly stained to the right Orcein fraction no 4 of Brolin & Hassler in association with van Gieson × 60

The changed elastic lamella had a normal appearance but was much thinner and more weakly stained than in the adjacent parts of the artery However, the adjacent parts of the artery had the lamella fragmented and split into two or three lamellae The media showed a moderate fibrosis which was not accentuated over the weakly stained part of the elastic lamella The findings in the intima and adventitia were largely the same as in case 2 although the extra external elastic layer was less pronounced

DISCUSSION

In an intracranial saccular aneurysm the two main layers of the cerebral arterial wall, i e the elastic lamella and the muscular media are missing, and the vessel wall is mainly built up of collagenous connective tissue The aetiology of these aneurysms is not known *Forbus* (1930) observed defects in the medial layer at the predilection sites of aneurysms Aneurysms are now generally believed to develop on the basis of a media defect However, these defects are very common, and most individuals have several As *Glynn* (1940) has pointed out, the elastic lamella alone is strong enough to cope with the load of the blood pressure

Several theories of "added factors" involving the elastic lamella have therefore been elaborated *Forbus*'s original conception, that the elastic layer gave way solely for an overstretching and exhaustion, has not been generally accepted *Richardson & Hyland* (1941) and *Kraulund* (1957) pointed out that aneurysm is not much more common in individuals with hypertension than in other subjects, and questioned whether the resistance of the elastic layer might not instead be impaired by toxic processes *Carmichael* (1950) and *Walker & Allègre* (1953) observed intimal thickening at the edges of the aneurysms which *Carmichael* regarded as being atheromatous in nature and responsible for the destruction of the elastic layer On the other hand *Glynn* (1940) found the destruction of the elastic lamella to be caused by a non atheromatous process The latter author went so far that he believed this added factor to be more important than the media defect



Fig 3

Higher power photomicrograph of the extra external elastic layer in Fig 1
Several round cells are seen in the adventitia × 120

media had a normal appearance Outside the media an external elastic layer could be seen just over the changed lamella but nowhere else in the preparation cf Fig 3 This extra layer consisted of a large number of very fine elastic fibres The adventitia over the changed lamella was considerably rich in round inflammatory cells Several although not many of these inflammatory cells were found in the surrounding parts of the adventitia and in addition many erythrocytes were found here because of the subarachnoidal bleeding The adventitia seemed also to be somewhat thicker than normal which probably also was the result of the subarachnoidal haemorrhage and the spastic contraction of the artery

Case 2

Male habitual drunkard 42 years

Principal macroscopical findings at autopsy Ruptured saccular aneurysm on the right internal carotid Subarachnoidal haemorrhage partly extending to the subdural space Slight atheromatous changes of the large cerebral arteries Weight of heart = 410 g

Microscopical findings Only one area with a changed elastic lamella was found in this case It was situated in the anterior communicating artery The size was about 0 05 mm²

The staining properties and the general appearance of the changed elastic lamella were largely the same as in case 1 The intima media and adventitia resembled also this case However the extra external elastic layer of case 1 was less pronounced and the round cell infiltration was less markedly increased over the changed lamella

Case 3

Male waiter aged 50

Principal macroscopical findings at autopsy Ruptured aneurysm of the right middle cerebral artery Enlargement of left side of heart Weight of heart = 520 g

Microscopical findings One area with a changed elastic lamella was found in the left anterior cerebral artery at the level of the anterior communicating artery (cf Fig 4) The size was about 0 05 mm²

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In an earlier paper (*Hassler* 1961) breaches in continuity of elastic lamellae were found to be more common in cerebral arteries from individuals with intracranial aneurysm than in other subjects This was especially the case with total ruptures of the internal elastic layer (Reuterwall's tears) which were healed by connective tissue The high incidence of tears was suspected to be caused by a lowered resistence of the elastic layer, *i e* the "added factor" of earlier workers Histological scrutiny of the Reuterwall's tears failed to disclose any pathological changes in the broken lamella The areas of weakly staining elastic lamella described in this paper may however be an expression of this added factor

In this connexion, the changes of the elastic lamella often seen at the margin of saccular aneurysms should be recalled They consist of increased elastin staining properties, diffuse swelling, eosinophilia, and fragmentation (*Hassler* 1961) Because such well-defined changes were *found only over media-defective areas of the wall, they were considered* to be secondary to the media defect, and regarded as a compensatory measure having nothing directly to do with an increased frequency of Reuterwall's tears

The extra external elastic layer at these changed areas should be noted As *Wolff* (1948) points out, this layer is normally seen only in the parts of the meningeal arteries facing the cranial bone I have also observed it over large atheroma and at the mouths of aneurysms

The histologist and pathologist have no exact methods for the demonstration of elastic tissue Several phases of the mechanisms behind the commonly used elastin-staining procedures remain obscure (*Brohn & Hassler* 1961) Nevertheless pathological processes in elastic tissue may play an important role for the development of arteriosclerosis and other important diseases The chemical composition of elastine is known to show great divergencies in various parts of the body and in different mammals (*Lansing* 1959) Among the few diseases of elastic tissue subjected to thorough investigation and well worth studying in the literature because of the techniques used, the Gronblad-Strandberg syndrome should be mentioned (*Carlborg, Ejrup, Gronblad, Lund & Lundmark* 1960) and the senile elastosis (*Braun Falco* 1956)

SUMMARY

In the large cerebral arteries from three patients with intracranial saccular aneurysms, areas with weak staining and thin elastic lamella have been observed These areas were accompanied by an extra external elastic layer and round cell infiltration The relationship between these abnormal areas and aneurysm-formation is discussed



Fig 1

at the embedding procedure Serial sectioning of the arterial segments was made Every second section of the series was chosen for the elastase digestion experiments while the remaining ones were treated with the same stains as the test sections after elastase digestion

METHODS

A *Elastase Treatment of Fixed Arteries*

The formaline was washed out by placing the arteries in running water for 24 hours The arteries were st[illegible]
elastase The solution wa[illegible]
amount of distilled water [illegible]
was done with distilled w[illegible] elastase (Worthington 2 X crystallized) was finally added The tubes were capped and [illegible]

[illegible] and treated with an elastin stain and counterstained with a general stain

B *Elastase Treatment of Sections on Slides*

The paraffine sections were deparaffinized in xylol and brought to distilled water via alcohol Droplets of the elastase buffer solution mentioned above were [illegible]

[illegible] solutions mentioned above

RESULTS

A *Digestion of the Fixed Arteries*

The internal elastic lamella of the human cerebral arteries did not as a rule take up any elastin stain after digestion When treated with van Gieson the digested lamella of young individuals did not take up any stain while that of old individuals stained weakly red like collagenous

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ELASTASE TREATMENT OF FIXED ARTERIAL ELASTIC TISSUE

By

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Received 17 viii 61

The elastic connective tissue differs in several respects from other tissues of the body. Its physical properties play a great role in the arteries, the skin etc. Little is known about the normal metabolism of the elastic tissue, but it is generally considered to be bradytrope, and nothing much is known either about the pathological processes in the elastic tissue, although they may play a great role in the development of arteriosclerosis, the mechanism of ageing etc.

The elastic tissue is very resistant towards chemicals and to boiling in dilute acids and alkali. Most enzymes have no effect on elastic tissue. An exception is an elastase discovered by *Balo & Banga* in 1950 which digests the elastic tissue.

A broad area of research was opened by the introduction of elastase. Much work has been carried out during the last years on the effect of elastase on purified elastin, and on fresh elastic tissue. Often, however, only fixed and perhaps also paraffine-embedded, sectioned material are available. In order to test the possibility of using such material the following experiments have been performed.

MATERIAL

A. *Segments of Arteries Fixed in Neutral Formaline*

1) Human cerebral arteries. The intradural portion of the left internal carotid and the most proximal parts of the anterior and middle cerebral arteries were taken from 10 individuals within 36 hours of death. The 10 individuals were chosen at random from the autopsy file of a hospital. Care had been taken only that each decade of age under 90 years was represented by two individuals.

2) Rabbit cerebral arteries. The circle of Willis with its large afferent and efferent intra dural branches was fixed from five rabbits immediately after the animals were killed.

3) Rabbit aorta. The thoracic and abdominal aortas from the same five rabbits were fixed as above.

B. *Paraffine Sections Mounted on Slides*

Sections 10 or 25 μ thick were taken from human cerebral arteries, rabbit cerebral arteries and rabbit aorta. The human arteries belonged to both adults and children. Formaline fixation was performed as above. Alcohol and xylol served as intermedia



Fig 1

Section through the middle cerebral artery of a 53 year old man The artery is fixed in formaline treated with elastase embedded and sectioned When subsequently treated with aldehyde fuchsin and van Gieson the internal elastic lamella stains red like collagenous connective tissue (× 160)

at the embedding procedure Serial sectioning of the arterial segments was made Every second section of the series was chosen for the elastase digestion experiments while the remaining ones were treated with the same stains as the test sections after elastase digestion

METHODS

A *Elastase Treatment of Fixed Arteries*

The formaline was washed out by placing the arteries in running water for 24 hours The arteries were st
elastase The solution wa
amount of distilled water
was done with distilled w
crystallized) was finally added The tubes were capped and placed horizontally in a shaker in water bath at 37 0° After 2 or 12 hours 5 0 ml of 0 5 M phosphate buffer (pH 6 0) was pipetted into each tube to stop the reaction The arterial segments were placed in neutral 5 per cent formaline for 36 hours after which they were embedded in paraffine with alcohol and xylol as intermedia 10 µ thick sections were produced and treated with an elastin stain and counterstained with a general stain

B *Elastase Treatment of Sections on Slides*

The paraffine sections were deparaffinized in xylol and brought to distilled water via alcohol Droplets of the elastase buffer solution mentioned above were applied to the h

RESULTS

A *Digestion of the Fixed Arteries*

The internal elastic lamella of the human cerebral arteries did not as a rule take up any elastin stain after digestion When treated with van Gieson the digested lamella of young individuals did not take up any stain while that of old individuals stained weakly red like collagenous

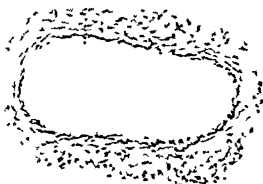


Fig 2

Rabbit cerebral artery This paraffine section has been deparaffinized and digested with elastase Afterwards the internal elastic lamella takes up no stain when treated with aldehyde fuchsin and van Gieson (× 150)

connective tissue (cf Fig 1) The normal internal structure of the lamella (cf Fig 2) could not be observed in the arteries from young individuals The lamella of old subjects showed a less homogenous picture Some of the internal structures disappeared, especially those showing strong elastin staining, and the fragmentation and splitting up of the lamella was more marked Under large intima cushions portions of the lamella were observed which had not been digested and took up elastin stain

The lamella seemed not to have thinned during the digestion procedure The borders to the media and the intima were distinct Fenestrations were visible

Findings in accordance with the ones seen in the human cerebral arteries and mentioned above were seen in the rabbit arteries However, the elastin of the external elastic layer of rabbit aorta was poorly digested (see Fig 3)

B *Digestion of Sections on Slides*

The elastin of the internal elastic lamellae was entirely dissolved The structure of the lamellae was largely the same as that of the elastase-treated arteries described above under A In some cases the external elastic layers of rabbit aorta took up small amounts of elastin stain in contrast to the internal elastic layers Also the fine elastic fibrils which occur in the media, the external elastic layer, and the adventitia of the cerebral arteries of young individuals were sometimes incompletely dissolved in contrast to the internal elastic lamella In the thick sections of rabbit aorta small pits were formed here and there, probably because small pieces of the sections had flaked off when the elastin had dissolved



Fig 3
Section through rabbit aorta treated with elastase after fixation The internal elastic layers do not take up much elastin stain in contrast to the external ones (Aldehyde fuchsin and van Gieson, × 100)

CONCLUSIONS

It is obvious that elastase dissolves the elastine of fixed, paraffin-embedded, arterial tissue The external shape of the elastic lamellae is not changed Some internal structures are eliminated while others stand out more clearly One reason why the elastine of the external elastic layer of the rabbit aorta had not dissolved regularly may be that the enzyme failed to reach this part of the arterial wall although it seems more probable that the internal parts of the media would be the ones less easily reached by the elastase solution Another possible explanation is that the elastine of these layers indeed is less digestable to elastase due probably to other physical or chemical properties A support for such a concept was also derived from the digestion experiments with paraffine-sections

Because after digestion, the elastic lamellae of old individuals stained weakly like collagenous connective tissue whereas lamellae of young individuals did not take up any stain, changes between the former and the latter lamellae were suggested Only formaline-fixed elastic tissue has been investigated but the effect of various fixative agents on elastic tissue is known to vary little (*Pearse* 1960)

SUMMARY

Elastase dissolves the elastine of formaline-fixed, arterial elastic tissue The elastine of paraffine embedded, sectioned arteries can also be dissolved by elastase After elastase dissolution, the elastic lamellae

of old individuals stain weakly like collagenous connective tissue whereas those of young subjects do not take up any stain After dissolution the external shape of the lamellae is not much altered Some internal structures are removed while others stand out more clearly The elastine of the external elastic layer of rabbit aorta and of some childrens' cerebral arteries were less readily digested

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PHYSIOLOGICAL INTIMA CUSHIONS IN THE LARGE CEREBRAL ARTERIES OF YOUNG INDIVIDUALS

I Morphological Structure and Possible Significance for the Circulation

By

OVE HASSLER

Received 13 IX 61

In the past much work has been done on sphincter mechanisms at the branching points of arteries other than the cerebrals It is necessary only to mention the very comprehensive investigations of *Julin* (1887) and *Vialleton* (1903) on cyclostomen, of *Dragendorff* (1911) on fishes and amphibia, *Mark* (1952) on birds, of *Legait & Legait* (1953) and *Waagenwort* (1954) on mammals, and of *Zink* (1941) on the coronary arteries of man *Rotter, Wellmer, Hinrichs & Muller* (1955) made an extensive investigation of the pathological changes which occur in those structures of the human cerebral arteries which may correspond to the sphincter mechanisms of other arteries The following work was started in order to investigate these structures in young children's arteries, where pathological changes are absent

MATERIAL AND METHODS

The large cerebral arteries from 63 individuals under the age of 20 years were investigated 34 of the individuals were less than one year old and 11 were 11 years or more The material was taken [illegible]
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A large num [illegible]
ones were sp [illegible]
the adjacent [illegible] distal portions of both anterior cerebral
arteries b) [illegible]
cm of the [illegible]
arteries c) [illegible]
situated ½ [illegible]
adjacent ½ [illegible]
embedded i [illegible] as possible parallel to the longitudinal axis of the trunk and the largest branch Serial sections were prepared and treated with various elastin and general stains

RESULTS

Several hundred cushions were observed Each cushion was covered with endothelium towards the arterial lumen and demarcated from the media by an elastic lamina (cf Fig 1)

TABLE 1

Structural Composition of Intima Cushions at Different Ages and in Different Arteries

Age	Artery	Cushion contents							
		Smooth muscle	Elastic fibrils	Elastic lamellae	Colla-genous connective tissue	Chromato-trope sub-stance	Nerve supply	Vasa vasorum	Muscular thickening under the cushion
< 1	Int car art & bas art	++	+++	(+)	(+)	(+)	+	(+)	++
	Ant cer & mid cer arts	+++	+	—	(+)	—	+	—	++
1 10	Int car art & bas art	++	++	+	(+)	+	+	+	++
	Ant cer & mid cer arts	+++	(+)	(+)	(+)	(+)	+	(+)	++
11 20	Int car art & bas art	++	(+)	++	+	++	+	+	+
	Ant cer & mid cer arts	+++	(+)	+	(+)	+	+	+	+
> 50	Int car art & bas art	(+)	(+)	+++	+++	+++	(+)	+++	—
	Ant cer & mid cer arts	+	(+)	++	++	++	(+)	++	—

The results of a previous investigation (*Hassler* 1961) on subjects aged more than 50 years are shown by way of comparison

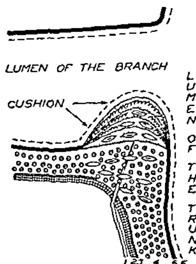


Fig 1

Diagram to illustrate schematically the structure of the cerebral arterial wall at

1 Periarterial nerve plexus
2 Adventitia
3 External elastic layer
4 Muscular media
5 Internal elastic lamella
6 Endothelium

Most cushions contained much elastic tissue Some of this tissue occurred in the form of thin, split up, elastic lamellae as is generally the case in intima cushions of adults (*Rotter, Wellmer, Hinrichs, & Müller* 1955 and others) The remainder of the elastic tissue was in the form of extremely fine elastic fibrils In the individuals aged 11 20 years most of the elastic tissue in the cushions was in the form of lamellae (see Table 1) On the other hand in young children, and especially in the new born most of the elastic tissue of the cushions occurred in the form of extremely fine elastic fibrils which were distributed diffusely throughout the cushion Some cushions of new born infants were mainly built up of such elastic fibrils (see Figs 2 *B* and 3 *B C*)

Smooth muscle cells were identified in most of the cushions In about half the muscle contents were estimated to comprise more than 25 per cent of the total volume, and in about one third the muscle contents appeared to take up more than 50 per cent of the total volume (cf Figs 2 *A* and 3 *A*) In one half of all cushions in which smooth muscle cells

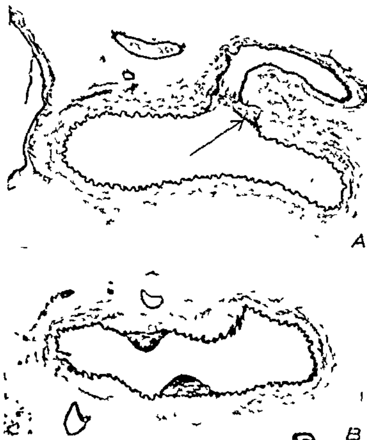


Fig 2

Sections through arteries with physiological intima cushions *A* Cushion (——→) in the middle cerebral artery mainly consisting of smooth muscle (× 40) *B* Two elastic cushions in the basilar artery (× 50) (Aldehyde fuchsin and van Gieson)

were identified, only a few scattered cells were found Proportionally more cushions with a high muscle content were found in the individuals aged 11–20 years than in the younger ones The nuclei of the muscle cells were arranged in a longitudinal direction (almost all muscle cells in normal cerebral arterial walls are arranged in low, nearly circular spirals) Most muscle cells were found in those parts of the cushion bordering on the media but separated from it by the elastic lamina In many cases epithelioid cells were observed which differed from the smooth muscle cells in having a pale cytoplasm and atypical nuclei These epithelioid cells seemed to be more common in the new-born The autolytic changes which are inevitable in post-mortem material often made differentiation of the epithelioid cells difficult, however

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Fig 3

Higher power photomicrographs of physiological intima cushions *A* Cushion mostly built up of smooth muscle *B* Cushion of mixed type *C* Cushion mainly built up of elastic tissue (Aldehyde fuchsin and van Gieson Magnification *A* × 95 *B* × 110 *C* × 110)







Fig 3

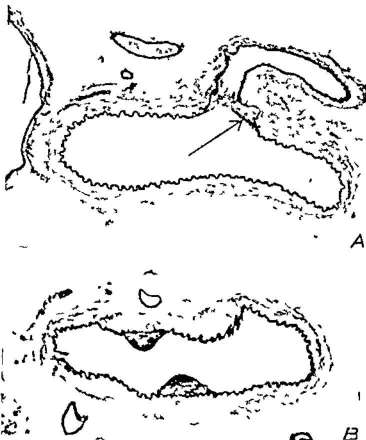


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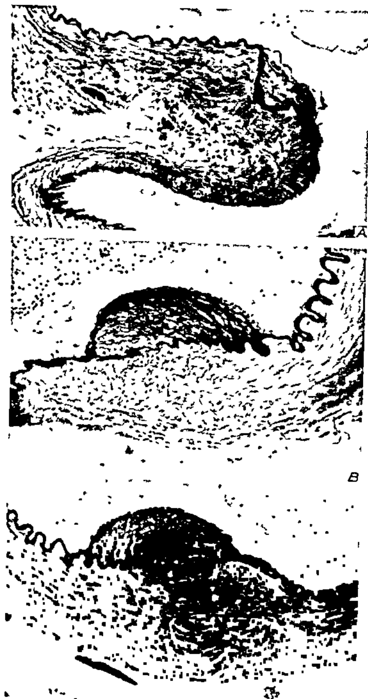


Fig 3



Fig 4

Section through the arterial wall under a cushion Vasa vasorum and a nerve (——→) with destinations to the cushion are seen (Aldehyde-fuchsin and van Gieson × 160)

Scattered fibrocytes and an extremely fine network of collagenous fibres were regularly present Chromatropic substance was demonstrated in several of the thickest cushions This was especially the case in the arteries from the oldest individuals of the series

Large nerves from the periarterial plexus were seen to enter the cushions in several cases (cf Fig 4) Vasa vasorum, which normally are observed only rarely in the cerebral arteries of man, except in intima cushions, could not with certainty be identified in new-borns In a large cushion from a 9-days-old boy a tiny vasa was found

The arterial wall under the cushions differed from the surrounding arterial wall in several respects The media contained layers of longitudinal muscle lying between the "normal" circular layers (see Figs 3 *B* and 3 *C*) Thus the total amount of smooth muscle at the site of the cushion was much increased in most cases Sometimes cushions were observed which were comparatively small but had a greatly thickened muscle layer below them In some cases there was no definite muscle-thickening, in five cases the muscle layer under the cushion was reduced One of the latter cushions was situated close to another large cushion, at the site of which there was a greatly thickened media The cushions in four of the five cases were situated at the terminal bifurcation of the internal carotid

The framework of connective tissue in the media under the cushions

also seemed to be strengthened (see Figs 3 C and 4) Each smooth muscle cell was enveloped in a thick layer of collagenous connective tissue containing scattered elastic fibrils Slight metachromasia was sometimes observed The external elastic layer of the artery was regularly absent over the cushions The adventitia was a rule thicker, with thick densely packed collagenous fibres

DISCUSSION

In the literature much confusion prevails concerning normal physiological, and pathological atheromatous intimal thickening of the cerebral arteries Most authors do not distinguish between two types, all kinds of intimal thickening being referred to as "atheromatosis" *Hackel* (1928) emphasised the difference, and stated that no fat is demonstrable in physiological intima thickening *Glynn* (1940) agreed with *Hackel*, and maintained that ' these localized intimal elastic hyperplasias develop through a process of work hypertrophy' An investigation into physiological intimal thickening was performed by *Rotter, Wellmer, Hinrichs & Muller* (1955), who found it to be localized regularly to branching points, and called it "Polster", a term which is probably best translated as intima cushion' Some of their intima cushions were found to contain muscle, while other cushions were mainly built up of elastic tissue *Rotter et al* assumed that the cushions function as sphincters and are important regulators of the peripheral resistance The investigation of *Rotter et al* was concerned mainly with pathological changes (fibrosis, hyalinosis, atheromatosis) of the cushions in hypertonia It was based on a series of only 20 individuals, of whom the youngest was 6 years, the majority being elderly individuals

I order to elucidate the functional significance of the intima cushions I have investigated their normal morphology Material as free as possible from atheromatosis and acquired arterial lesions is obtained from newborn infants and children A possible objection to such an investigation in children is the incomplete development of the arteries Several comparatively large cushions were found even in the arteries of neonates however

The significance of the cushions may lie in the fact that they can influence the blood flow to a branch and increase the peripheral resistance The extent to which the cushions protude into the lumen can probably be regulated by the longitudinal muscle in and under the cushion This muscle makes the cushion shorter and thicker during contraction Arteries showing marked post mortem contraction of the wall (*Hassler* 1962) provide further evidence in support of this view, as may also the abundant innervation which was observed There are certain points which do not favour this view, however Firstly, the muscle layer is not always thickened at the site of intima cushions, in some cases it is reduced or totally lacking This seems to be mostly

the case in elderly individuals with fibrotic cushions Secondly, the cushions observed are not circular, if they had enclosed the branch-origin, they would probably have been more effective as sphincters

The high elastic tissue content is less easily explained It may possibly modify the pulsations from the heart, and operate as a "Windkessel"-mechanism The large cushions, containing much elastic material, may also cause a water-hammer effect (*Gibson* 1919, *Muller* 1935), or give rise to pulse-wave reflexion and interference (*Wiggers* 1952, *Mc Donald* 1960, *Wehn* 1961) They may also cause turbulence distal to them, and give rise to aneurysms (*Hassler* 1961) The cushions are subject to fibrosis earlier in life than is the case of the surrounding arterial wall They may therefore be assumed to be very delicate structures, having a higher rate of metabolism than the surrounding wall

SUMMARY

Cushion-like structures are described in the intima of the cerebral arteries of children The cushions are situated at the points of branching They are mainly built up of longitudinal muscle and elastic tissue, and are richly supplied with nerves The cushions may regulate the blood flow through the branch, and cause an increase in the general peripheral resistance of the individual The cushions may also give rise to water-hammer effect and pulse-wave reflexion proximal to them and to turbulence distally to them

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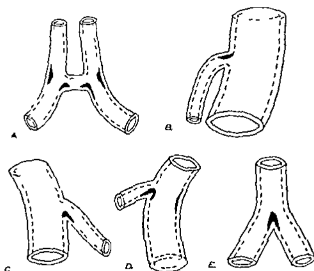


Fig 1.

artery departs from the internal carotid *D* The terminal bifurcation of the internal carotid to form the anterior and middle cerebral arteries *F* The confluence of the vertebral arteries to form the basilar artery

and this may cause disease and degeneration in them which in turn influences the circulation in Heubner's artery Because of their position, they may also cause an increase in the blood pressure in Heubner's artery, whereas other cushions probably cause a lowering of the blood pressure in their respective branches The cushions at the origin of Heubner's artery may also cause a water-hammer effect and pulse-wave reflexion into the artery This unique function of the cushion in Heubner's artery may have some connection with the recurrent course of the artery The origins of other recurrent arteries, *e g* the lateral striate arteries were studied in a few cases without any cushions being observed Since initially no investigation of the origins of other recurrent arteries had been envisaged, no systematic examination of these was carried out

SUMMARY

Because of the clear-cut and well-defined histological appearance of the intima cushions of the cerebral arteries of children, detailed diagrams of the location of the cushions have been worked out The position of the cushion at the origin of Heubner's recurrent artery differs notably from that of other cushions in that it is not sheltered from the main blood stream It may then cause an increase instead of a decrease in the blood pressure in Heubner's artery It may also cause a water-hammer effect and pulse-wave reflexion into Heubner's artery

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PHYSIOLOGICAL INTIMA CUSHIONS IN THE LARGE CEREBRAL ARTERIES OF YOUNG INDIVIDUALS

2 *Location*

By

OLF HASSLER

Received 13 IX 61

MATERIAL AND METHODS

See the first paper (*Hassler* 1962) of this series

RESULTS AND CONCLUSIONS

The observations concerning the location of the cushions in children are largely in accordance with those made in adults in connection with an earlier investigation (*Hassler* 1961) Diagrams showing their exact position could easily be made (cf Figs 1 A–E), since there was no generalized intimal thickening in the children, and because the cushions were clear cut with no atheromatous changes or fibrosis Cushions were *never observed at locations other than those indicated in the diagrams*

When cushions were observed in the anterior cerebral and anterior communicating arteries they were present at all four locations in about one third of the cases In the remaining two thirds there were either extremely small cushions or marked assymmetry of the H-shaped junction

No observations concerning the location of intima cushions in Heubner's *recurrent artery were made in the previous investigation*, because this was largely based on middle-aged and elderly subjects In such individuals the cushions are voluminous as a result of atheromatous and fibrotic changes In consequence, the cushions in the anterior cerebral artery at the level of the anterior communicating artery often surround the tiny origin of Heubner's artery *and become confluent with* its cushion, in other cases a diffuse intimal thickening in the anterior cerebral artery makes exact identification of the tiny cushions in the origin of Heubner's artery impossible

The location of the cushions observed in Heubner's artery of four cases is worthy of note These cushions are not sheltered from the main blood stream, as other cushions are (cf *Hassler* 1961) They may therefore *be subjected to more haemodynamic stress than other cushions*,

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PHYSIOLOGICAL INTIMA CUSHIONS OF THE LARGE CEREBRAL ARTERIES

3 *Individual Distribution among Different Ages and Arterial Groups*

By

OVE HASSLER

Received 13 ix 61

MATERIAL AND METHODS

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TABLE 1

Individual Distribution of Large and Small Cushions over Different Arterial Groups

Location	Ant com art	L mid cer art	R mid cer art	L int car art	R int car art	Bas art
Large cushions	2	1	2	5	4	4
Small cushions	12	13	15	24	20	25
Individuals with at least one large cushion	2	1	2	4	4	3
Individuals with no large cushion but at least one small cushion	6	7	9	11	10	12
[illegible]	55	55	52	48	49	48
[illegible]	63	63	63	63	63	63
[illegible] artery only	0	0	0	3	1	2
Individuals with large or small cushion in this artery only	1	2	0	5	6	7

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Individuals with no large cushion but at least one small cushion	6	7	9	11	10	12
Individuals with no large or small cushion	55	55	52	48	49	48
Total number of individuals	63	63	63	63	63	63
Individuals with large cushion in this artery only	0	0	0	3	1	2
Individuals with large or small cushion in this artery only	1	2	0	5	6	7

RESULTS

As shown in Table 1, more cushions with a large volume were found in the largest of the intracranial arteries, i.e. the internal carotid and the basilar arteries.

TABLE 2

Distribution of Large and Small Cushions among Different Age Groups

Age	< 1 year	1–10 years	11–20 years	Total
Total number of individuals	34	18	11	63
Large cushions	1	8	9	18
Small cushions	9	61	39	109
Individuals with at least one large cushion	1	6	7	14
Individuals with at least one small cushion, but no large one	5	10	3	18
Individuals with no large or small cushion	28	2	1	31
Large and small cushions per individual	0.3	3.8	4.4	2.0

The length and area of attachment seemed also to be closely related to the calibre of the artery. Cushions having a large volume were more common in older individuals than in younger ones (cf. Table 2). On the other hand, cushions having a large relative protrusion (a property thought to have greater functional significance than the volume) were not much more common in older individuals than in younger ones (see Table 4). Nor were such protruding cushions found more frequently in arteries of large calibre (cf. Table 3).

TABLE 3

Distribution of Markedly and Slightly Protruding Cushions over Different Arterial Groups

Location	Ant com art	L mid cer art	R mid cer art	L int car art	R int car art	Basilar art
Markedly protruding cushions	6	4	6	3	4	3
Slightly protruding cushions	34	26	30	19	16	14
Individuals with at least one markedly protruding cushion	4	2	3	3	2	3
Individuals with no markedly protruding cushion but at least one slightly protruding cushion	17	12	15	8	8	7
Individuals with no markedly or slightly protruding cushion	42	49	45	52	53	53
Total number of individuals	63	63	63	63	63	63
Individuals with markedly protruding cushion in this artery only	2	1	2	1	1	0
Individuals with markedly or slightly protruding cushion in this artery only	6	4	5	0	1	0

The individual variations were much greater than the age variations due to the physiological growth of the arteries Certain variations between different branch origins were also observed The cushions of the internal carotid and the basilar artery were generally found to contain more elastic tissue and less muscle than those of the anterior cerebral and middle cerebral arteries

TABLE 4

Distribution of Markedly and Slightly Protruding Cushions at Different Ages

Age	< 1 year	1 10 years	11 20 years	Total
Markedly protruding cushions	12	8	6	26
Slightly protruding cushions	65	41	33	139
Individuals with at least one markedly protruding cushion	5	4	2	11
Individuals with at least one slightly protruding cushion but no markedly protruding one	25	12	8	45
Individuals with no markedly or slightly pro [illegible]	4	2	1	7
	34	18	11	63
	2 3	2 7	3 5	2 6

A great number of cushions were observed which were too small to warrant classification They were excluded owing to difficulties in demarcation and confirmation when they occurred in only a few sections No important difference as to location or histological structure was observed in these small cushions when they were encountered It should be noted that the two types of classification did not always exclude the same cushions

CONCLUSIONS AND SUMMARY

Intima cushions are frequently found in the cerebral arteries of neonates As a rule, the cushions have a smaller volume in newborn infants than in older children The functional significance of the cushions is probably more closely related *to the relative* protrusion defined as the thickness of the cushion divided by the internal diameter of the artery, than to the actual volume The cushions from the neonates showed almost the same degree of relative protrusion as those from the older children The cushions of the largest arteries generally had a *greater* volume and a greater area of attachment but no greater [illegible]

[illegible] variations arising from physiological growth of the arteries *It is suspected that the great* [illegible]

[illegible] question [illegible]

RESULTS

As shown in Table 1, more cushions with a large volume were found in the largest of the intracranial arteries, i.e. the internal carotid and the basilar arteries.

TABLE 2

Distribution of Large and Small Cushions among Different Age Groups

Age	< 1 year	1–10 years	11–20 years	Total
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Individuals with at least one small cushion but no large one	5	10	3	18
Individuals with no large or small cushion	28	2	1	31
Large and small cushions per individual	0.3	3.8	4.4	2.0

The length and area of attachment seemed also to be closely related to the calibre of the artery. Cushions having a large volume were more common in older individuals than in younger ones (cf. Table 2). On the other hand, cushions having a large relative protrusion (a property thought to have greater functional significance than the volume) were not much more common in older individuals than in younger ones (see Table 4). Nor were such protruding cushions found more frequently in arteries of large calibre (cf. Table 3).

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Individuals with no markedly or slightly protruding cushion	42	49	45	52	53	53
Total number of individuals	63	63	63	63	63	63
Individuals with markedly protruding cushion in this artery only	2	1	2	1	1	0
Individuals with markedly or slightly protruding cushion in this artery only	6	4	5	0	1	0

FROM THE SECOND DEPARTMENT OF MEDICINE UNIVERSITY OF HELSINKI
(HEAD PROF ILMARI VARTIAINEN M.D.)

ON THE ANTITOXIC EFFECT OF VITAMIN B_{12}

By

W J KAIPAINEN

Received 10 ix 61

Penicillin (1) and erythromycin (3) are known to be toxic to guinea pigs The exact reason why antibiotics have this effect in guinea pigs remains unexplained although comprehensive studies have been carried out especially of penicillin Several theories have been advocated to explain the toxicity of penicillin but a generally acceptable theory has not been found Likewise numerous efforts to counter the toxicity of penicillin have failed The present writer (2) using rumen extract succeeded in preventing penicillin toxicity in guinea pigs As rumen extract contains innumerable and highly varied substances it was not endeavoured in the earlier work to establish which one of the constituents in the rumen extract represented the active component

As vitamin B_{12} plays an essential role in both human and animal nutrition experiments were performed to ascertain the influence of vitamin B_{12} in the prevention of penicillin and erythromycin toxicity in guinea pigs

MATERIAL, METHODS AND RESULTS

The guinea pigs were given 100 000 units of penicillin intraperitoneally on two successive days Vitamin B [illegible] ultane [illegible] B_{12} was [illegible] within [illegible] receiving 7 micrograms of vitamin B_{12} daily all of the animals survived The result was less satisfactory using a smaller dose and 0 1 microgram per os failed to exert any lasting influence (A dose of 7 micrograms corresponds largely to a dose of 1 000 micrograms in a man) Notwithstanding the favourable end result all of the animals were uniformly affected both the control group and the vitamin B_{12}-treated group Hence it was not definite that vitamin B_{12} was able expressly to eliminate the effect of [illegible]

[illegible] were rapid

As there is a certain close relationship between vitamin B_{12} and the intrinsic factor in the sense that intrinsic factor is necessary for the

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MATERIAL, METHODS AND RESULTS

The guinea pigs were given 100 000 units of penicillin intraperitoneally on two successive days Vitamin B_{12} was administered simultaneously by different routes The results are tabulated No vitamin B_{12} was given to the control group In this group all of the animals died within two weeks In the group receiving 7 micrograms of vitamin B_{12} daily all of the animals survived The result was less satisfactory using a smaller dose, and 0 1 microgram per os failed to exert any lasting influence (A dose of 7 micrograms corresponds largely to a dose of 1,000 micrograms in a man) Notwithstanding the favourable end result, all of the animals were uniformly affected, both the control group and the vitamin B_{12}-treated group Hence it was not definite that vitamin B_{12} was able expressly to eliminate the effect of [illegible]

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As there is a certain close relationship between vitamin B_{12} and the intrinsic factor in the sense that intrinsic factor is necessary for the

uptake of vitamin B_{12} from the alimentary tract, a control experiment was conducted to find the effect of intrinsic factor from pigs if given alone per os. Two commercial intrinsic factors were used and, as will be seen from the table, all of the animals died like the guinea pigs in the control group.

In addition to the above series, an investigation was carried out using erythromycin instead of penicillin. The procedure applied differed in that vitamin B_{12} administration was not commenced until the 6th day after the administration of erythromycin. By this time all of the guinea pigs were already very ill and dying, they were sitting or lying, were unresponsive, eating nothing, moulting, and lost about a fourth of their body weight; they would lick their loose stools. Under the influence of vitamin B_{12} the animals rallied considerably and became animated for about 4 hours, upon which they gradually withdrew into their corners again. The doses administered on the following days had a similar effect. However, medication had been started rather late and the guinea pigs were so ill that despite the vitamin B_{12} administration only 6 of the 10 animals survived for periods longer than 2 weeks and a further 2 died during the ensuing 2 weeks. In the control group 8 guinea pigs died on the 6th and 7th day, the remaining 2 died later.

TABLE 1

Penicillin and Erythromycin Induced Toxicity in Guinea Pigs Treated by Different Methods

No. of animals	Weight g	Toxicity induced by	Mode of treatment	Survivors after 1st	2nd	4th week
5	350–400	Benzylpenicillin 100 000 I.U. 2 days i.p.	Vitamin B_{12} 7 micrograms i.p. daily	5	5	5
5	380–400		Vitamin B_{12} 0.1 microgram daily i.p.	4	4	4
5	300–350		Vitamin B_{12} 0.1 microgram per os daily	4	1	0
18	320–600		No treatment	14	0	0
5	300–340		Intrinsic factor unpurified 0.1 gm daily per os	5	0	0
5	300–330		Intrinsic factor purified 0.3 mgm daily per os	5	0	0
10	350–400	Erythromycin 100 mg per os 5 days	Vitamin B_{12} 1 microgram daily from 6th day (2 weeks)	10	6	4
10	300–430		No treatment	2	0	0

DISCUSSION

No satisfactory explanation has yet been offered concerning the toxicity of penicillin and erythromycin to guinea pigs Rumen extract excepted no drug has been capable of countering a penicillin toxicity in guinea pigs The present work shows however that vitamin B_{12} has a distinct antitoxic influence on guinea pigs poisoned with these antibiotics

In these experiments however vitamin B_{12} could not prevent completely the toxic effect of penicillin The guinea pigs fell ill in spite of the vitamin B_{12} administration

It is worth mentioning in this connection that a patient was under treatment in the hospital at that time on account of methyl alcohol poisoning His vision deteriorated every day and had progressed as far as barely finger counting at 1 metre An intramuscular course of 1 000 micrograms of vitamin B_{12} twice a day was instituted and the patient s vision immediately began to improve after one week it was almost normal Another similar patient also presenting progressive symptoms of incipient blindness came to the hospital The case was managed in the same way and the result was a surprisingly rapid restoration of vision As blindness caused by methyl alcohol passes off spontaneously in 25 50 per cent (6) of the cases the role of vitamin B_{12} cannot be over stressed The cases are mentioned here with a view to suggesting that it might be possible to try the effect of vitamin B_{12} in other similar cases as well especially since no other substance is available by which a curative effect is obtainable in a corresponding situation

It is generally admitted that the liver is an organ which is capable of weakening the potency of poisons and drugs and on the other hand that this organ actually stores vitamin B_{12} Hence it would hardly be surprising if vitamin B_{12} were one of the agents in the liver providing the above property In large therapeutic doses exceeding storage capacity of the liver vitamin B_{12} might produce an antitoxic effect also peripherally in the tissues subject to the effect of the poison e g nerve [illegible] succeeds in normalising the blood values It may also be mentioned that some workers have regarded the toxic effect of penicillin on guinea pigs as a direct neurotoxic action (4 5 7)

SUMMARY

It has been shown that vitamin B_{12} has a counter effect on the fatal toxicity caused by penicillin and erythromycin in guinea pigs The rôle of vitamin B_{12} as an antitoxic agent in the liver is briefly discussed Two cases of methyl alcohol poisoning in man involving progressive loss of vision successfully treated by vitamin B_{12} are mentioned

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VIROLOGICAL DEPARTMENT OF THE BACTERIOLOGICAL INSTITUTE AND THE PATHOLOGICAL INSTITUTE UNIVERSITY OF LUND SWEDEN

INFECTIOUS FIBROMA IN PREDNISOLONE-TREATED RABBITS

By

SVEN BERGMAN, NILS JONSSON and C G AHLSTRÖM

Received 13 x 61

The infectious rabbit fibroma is of a transient nature and has an ambiguous anatomical character with a mixture of inflammatory and neoplastic features Therefore it was considered worth while to investigate whether treatment of the rabbits with cortisone was capable of tipping the balance between inflammatory and neoplastic components of the fibroma in favour of the latter

Harel & Constantin (6) injected fibroma virus intratesticularly and subcutaneously into normal and into cortisone-treated rabbits and found that the fibroma lesions were larger and persisted for a longer time in the animals treated with cortisone Sometimes "metastases" appeared, and in one case the tumour had the histological appearance of a sarcoma

In the present investigation fibroma virus was injected intracutaneously or intravenously into normal and into prednisolone-treated rabbits The sizes of the cutaneous fibroma lesions may be determined rather precisely In normal rabbits intravenous injection of the fibroma virus will produce no reaction apart from a small fibroma developing at the site of injection and occasionally a few small pocks in the skin In addition, both the prednisolone-treated and the untreated rabbits were studied for antibodies after infection with the virus

MATERIAL AND METHODS

The fibroma virus (*Patuxent strain*) was kindly supplied in vacuum dried state by Sir *Christopher Andrewes* Institute of Medical Research London Rabbits were inoculated intratesticularly with the virus and infected testis tissue was finely cut with scissors ground with powdered glass and [illegible]

[illegible]

[illegible] of virus neutralizing antibodies was carried out by mixing equal parts of rabbit serum in different dilutions with 100 TCD_{50} of the fibroma virus from a pool cultivated on embryonal rabbit fibroblasts This mixture was incubated for 1 hour at +37° C, after which 0 1 ml was inoculated into tube cultures of rabbit fibroblasts 5 tubes being used for each dilution The results were appraised every other day until the day after the virus controls showed a distinct cytopathogenic

effect. The highest virus neutralizing serum dilution was noted. The cytopathogenic effect was readily recognized and appeared on the fifth or sixth day.

Prednisolone was given in the form of Precortalon (Pharmacia) intramuscularly or subcutaneously.

Most of the rabbits received altogether 50–75 mg of prednisolone in 3 to 5 doses given at intervals of 4 to 6 days. The first dose was given on the same day as the fibroma virus. Some animals received only 20 mg of prednisolone. The rabbit studied for virus neutralizing antibodies received a weekly dose of 25 mg prednisolone for 6 weeks.

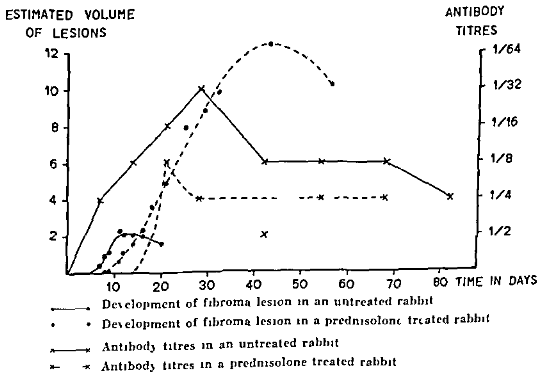
RESULTS

1. *Intracutaneous Inoculation of Prednisolone-Treated Rabbits with Fibroma Virus*

Twelve prednisolone-treated rabbits were inoculated intracutaneously with the fibroma virus along with normal untreated controls. Four of the prednisolone-treated animals received 0.1 ml of a testis suspension diluted 1:10 to 1:10^7, while the remaining 8 rabbits were given 0.25 ml of a 1:1000 suspension at 1–4 sites.

In the prednisolone-treated animals the reaction of the skin appeared 1 to 2 days later than in the controls. In both groups a dilution of 1:10[illegible] –1:10[illegible] gave a positive reaction and no difference in susceptibility to the virus was observed. Initially the skin tumours increased rapidly in size. On the tenth to twelfth day the lesion in the controls showed the usual signs of regression: superficial haemorrhages, a central depression, and a reduction in size. In the prednisolone-treated animals, on the other

DIAGRAM 1





Figs 1 2

Fig 1 Fibroma 16 days after inoculation with the fibroma virus in prednisolone treated rabbit Htx eosin × 480

Fig 2 Fibroma 27 days after inoculation with the fibroma virus in prednisolone treated rabbit Htx eosin × 480

hand, the tumours retained their vital appearance for one month or more (Diagram 1) The tumours were of firm consistency, they were well defined and showed a smooth, pink surface with only scanty haemorrhages on the top of the tumour When regression finally started, it progressed relatively slowly

Histologically, the infectious rabbit fibroma is built up of a fibromyxomatous tissue with an admixture of inflammatory cells, which increases with the age of the lesion In the prednisolone-treated animals the picture differed in so far as the inflammatory cells were missing or were very scanty until the tumour regressed Hence, the appearance of the lesions resembled that of a true neoplasm more than otherwise On the ninth day after inoculation the tumours showed a loose, fibromyxomatous structure The picture remained largely the same 1 week later (Fig 1), when the lesions in the control animals had been shed completely During the following weeks the tumours assumed a more cellular character (Fig 2), the cytoplasm in the cells became more abundant and the cells were more crowded than before Staining revealed PAS-positive dropets in many of the cells

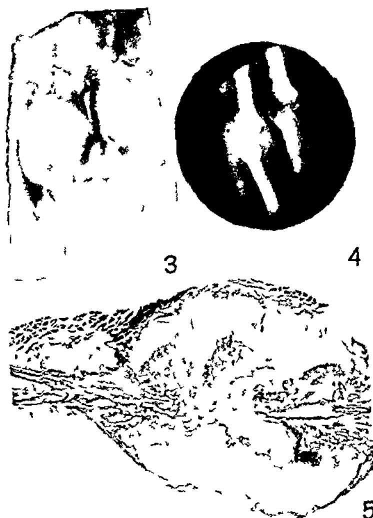
During the first 2 days after intracutaneous inoculation with the virus the rabbits not treated with prednisolone showed a circumscribed oedematous loosening of the subepithelial tissue as well as incipient proliferation of large connective cells, the fibroma cells In the prednisolone-treated animals the serous exudation was initially markedly inhibited, and the proliferation of the fibroblasts appeared to be retarded

In the prednisolone-treated animals the epidermis over the fibroma nodules was hyperplastic and showed epithelial projections extending downwards into the fibromatous tissue Many of the epidermal cells were vacuolated The epidermal changes over the lesions in rabbits which had not received prednisolone were much less prominent

2 *Intravenous Inoculation of Prednisolone-Treated Rabbits with Fibroma Virus*

Seven prednisolone treated rabbits and 2 untreated controls received 1 ml of a 1 1000 testis suspension intravenously The animals were killed one month later The two controls showed nothing remarkable Two of the prednisolone-treated animals developed generalized fibromatosis The lesions were localized to the ribs, where they produced a fusiform or nodular, greyish-white firm swelling (Fig 3) Within the swelling the bone was destroyed (Fig 4) and, microscopically, scattered islands of fibroma tissue with signs of regression could be seen (Fig 5) within a fibrous, partly cartilaginous callus One of the rabbits showed, in addition to rib tumours, a peasized fibroma in the abdominal musculature

Ahlstrom & Andrewes (1) have shown that intravenous injection of



F gs 3 5

F g 3 Fibr ma lesions localized to ri s of predn sol ne treated rabbit inoculated

has begun and callus has formed in fractured region Peripherally

fil roma virus into rabbits treated with tar or carcinogenic hydro carl ons gives rise to generalized fil romatosis and sometimes to a localization of the virus at the site of the injection of the tar In one of their animals a progressively growing sarcoma developed (2) In the light of this it was considered reasonable to study the effect of the fi broma virus on rabbits treated with both carcinogenic hydrocarl ons and cortisone

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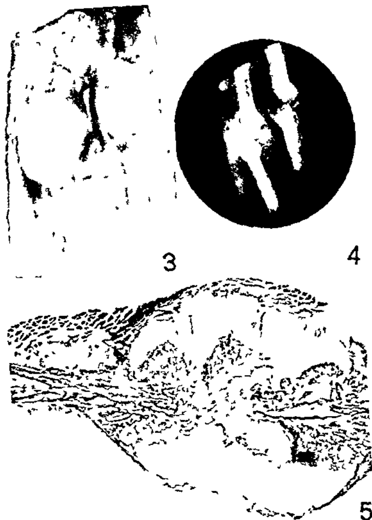
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Figs 3 5

Fig 3 Fibroma lesions localized to ribs of prednisolone treated rabbit inoculated intravenously with fibroma virus

Fig 4 Rib fractured at site of fibroma Healing has begun and callus has formed

Fig 5 Residual fibroma tissue is seen centrally in fractured region Peripherally the tumour is made up of cartilage-callus

fibroma virus into rabbits treated with tar or carcinogenic hydrocarbons, gives rise to generalized fibromatosis and sometimes to a localization of the virus at the site of the injection of the tar In one of their animals a progressively growing sarcoma developed (2) In the light of this it was considered reasonable to study the effect of the fibroma virus on rabbits treated with both carcinogenic hydrocarbons and cortisone



Fig 6

Fibroma at limbus corneae in rabbit inoculated intravenously with fibroma virus and treated with prednisolone and 9 10 dimethyl 1 2 benzanthracene



Figs 7–8

Fig 7 Large fibroma over spine and small subcutaneous fibroma in a rabbit inoculated intravenously with fibroma virus and treated with prednisolone and tar

Fig 8 Rounded fibroma at edge of right orbit of same animal as in Fig 7

Twelve rabbits, of which six were treated with prednisolone, received 0 2 0 5 ml of tar (Horizontal Retort Tar from Leeds) intramuscularly into the right thigh On the same day all of the animals were inoculated intravenously with the fibroma virus Generalized fibromatosis developed in 4 of the 6 rabbits receiving tar exclusively, and in 4 of the animals treated with both tar and prednisolone Three of 5 prednisolone-treated rabbits receiving intramuscular injections of 1 ml of 9,10 dimethyl-1,2 benzanthracene in olive oil also developed generalized

fibromatosis In many of the animals the virus accumulated at the site of injection of the tar or dimethyl benzanthracene, where it gave rise to rapidly growing firm infiltrates which, however, regressed spontaneously The generalized lesions were seen in the ribs, conjunctiva (Fig 6) roof of the skull (Fig 8), beneath the skin and over the spine (Fig 7) Histologically, they showed a fibromyxomatous picture with only scanty inflammatory cells In no instances were any progressively growing tumours observed

Antibody Formation in Prednisolone Treated Rabbits Inoculated with Fibroma Virus

In an experimental series of 20 adult rabbits (weight 2 500–3 500 g), divided into 2 groups 16 of the animals received 3 doses of testis suspension diluted 1 10 1 100 and 1 1000 Eight of these 16 animals were treated with prednisolone Of the remaining four, 2 received no treatment at all and 2 were treated with prednisolone only

As in the other experiments the growth of the fibromas was initially inhibited and the tumours usually attained larger sizes and persisted much longer in the prednisolone treated rabbits (Diagram 1) During the experimental period the prednisolone treated animals lost weight as a rule by 25 per cent

Venous blood was collected at weekly intervals for at least 6 weeks The titres of the virus neutralizing antibodies in the 2 groups are given in Table 1 and 2 respectively A typical example of the development of the lesions (dilution 1 100) and the shape of the antibody curve is given in Diagram 1

TABLF 1

Highest Virus Neutral ing Serumdilution

Treatment	Rabbit No	Days after inoculation					
		0	7	11	21	28	42
None	1	<1 4	<1 4	<1 4	<1 4	<1 4	<1 4
	2	<1 2	<1 2	<1 2	<1 2	<1 2	<1 2
Prednis lone	3	<1 2	<1 2	<1 2	<1 2	<1 2	<1 2
	4	<1 2	<1 2	<1 2	<1 2	<1 2	<1 2
Fibroma virus	5	<1 4	1 32	1 32	1 32	1 64	1 64
	6	<1 4	1 4	1 16	1 16	1 64	1 64
	7	<1 4	1 4	1 16	1 32	1 64	1 128
Fibroma virus + prednisolone	8	<1 4	1 4	1 16	1 32	1 8	–
	9	<1 4	<1 4	1 8	1 16	1 32	1 64
	10	<1 4	<1 4	1 8	1 8	1 16	1 16

Serum from normal rabbits or rabbits treated with prednisolone only, never showed any measurable amount of virus neutralizing anti



Fig 6
Fibroma at limbus corneae in rabbit inoculated intravenously with fibroma virus and treated with prednisolone and 9,10 dimethyl-1,2 benzanthracene



Figs 7–8
Fig 7 Large fibroma over spine and small subcutaneous fibroma in a rabbit inoculated intravenously with fibroma virus and treated with prednisolone and tar
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bodies In the animals inoculated with fibroma virus antibodies were demonstrable 1 week later upon which the titre continued to rise for a further 3 weeks During the sixth to twelfth week the titre became stable at a low level In serum from animals treated with fibroma and prednisolone antibodies could not be demonstrated until 2 or 3 weeks after the inoculation The antibody curve rose during the fifth to seventh week to reach a peak 1 to 2 dilution steps below the one noted for animals not treated with prednisolone

DISCUSSION

It is well known that the reaction of rabbits to the fibroma virus is influenced by factors prevalent in the host In rabbits treated with tar or carcinogenic hydrocarbons the fibroma is seen to persist for much longer periods and to assume much larger proportions than in untreated animals (1) The same effect is obtained if the resistance of the animals is depressed by roentgen ray irradiation (3) The reaction also varies parallel with the age of the animals In newborn rabbits (4 5) the fibroma virus may give rise to a rapidly fatal generalized disease involving mainly destructive and inflammatory lesions Even if the newborn rabbits were inoculated with only small doses of virus rapidly growing solitary or multiple sarcoma like tumours will develop and often kill the animals

In our experiments the cortisone proved to influence the fibroma in two ways

The short but distinct initial inhibition of the reaction to the fibroma virus is probably due to the inhibitory effect of the cortisone on the exudation and possibly to some extent also to the power of the drug to counteract proliferation of the fibroblasts

The other effect of cortisone was to delay the onset of the regression of fibroma lesions as compared with findings in the untreated controls In untreated rabbits the regression of the fibroma is due to the death of cells affected by the virus and to the formation of viral antibodies which prevent infection of new cells The absence in the prednisolone treated rabbits of demonstrable viral antibodies until 2 3 weeks after the injection of the fibroma virus together with the fact that the highest value noted was much lower than the one found in animals not treated with prednisolone probably explains why the regression of the lesions was retarded It is possible that the antiphlogistic effect of the cortisone also contributed to the persistence of the lesions and that the direct attack of the virus on the cells had weakened the fibroma cells in the cortisone treated animals appeared to be well preserved even long after the lesions in the control animals had disappeared

It was noteworthy that the size of the fibroma appeared to vary with the amount of prednisolone administered The largest amounts were given to animals the antibody curves of which had been plotted

TABLE 2

Highest Virus Neutralizing Serum Dilution

Treatment	Rabbit No.	Days after inoculation								
		0	7	14	21	28	42	54	68	82
Fibroma virus	11	<1 2	1 2	1 16	1 8	1 8	1 4	1 8	1 4	1 2
	12	<1 2	1 4	1 4	1 32	1 16	1 16	1 16	1 8	1 8
	13	<1 2	1 4	1 8	1 16	1 32	1 8	1 8	1 8	1 4
	14	<1 2	1 8	1 4	1 8	1 4	1 4	1 4	1 8	1 4
	15	<1 2	1 2	1 4	1 4	1 4	1 4	1 4	1 2	1 2
Fibroma virus + prednisolone	16	<1 2	<1 2	1 4	1 8	1 16	1 16	1 4	1 4	–
	17	<1 2	<1 2	1 4	1 8	1 4	1 2	1 8	1 2	1 2
	18	<1 2	<1 2	<1 2	1 2	1 2	1 2	1 2	1 4	1 2
	19	<1 2	<1 2	<1 2	1 8	1 4	1 2	1 4	1 4	–
	20	<1 2	<1 2	<1 2	1 4	1 4	1 8	1 4	1 4	–

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SKELETAL ANGIOMATOSIS

Report of Two Cases

By

HARLAN J SPJUT[1] and AKE LINDBOM

Received 13 xi 61

Angiomas, either haemangiomas, lymphangiomas or combinations, involving multiple bones are rare as judged from reports in the medical literature (1–14) We have found 15 cases in the literature, including one illustrated by *Aegerter* (2) but not described in detail (Table 1) The evolution of the skeletal lesions has only been hinted at, because the follow-up of the reported cases has been relatively short, from no follow-up to 11 years One patient with multiple lymphangiectases was followed until autopsy an observation period of 6 years (3) We are reporting two patients with multiple skeletal angiomas, one followed for 32 years and one for 12 years Serial roentgenograms had been taken in both cases, and in one several biopsies were made

CASE REPORTS

Case 358 Bone Tumor Registry Radiopatologiska Institutionen The patient a 27 year-old woman was admitted to the hospital in November, 1948 because of a fracture of the right tibial condyle During the 10 previous years she had been suffering from episodes of fainting and cardiac arrythmias Roentgen examination revealed cystic alterations of the proximal and distal parts of the right tibia and of the distal metaphysis of the [illegible]
calcium was 9 9 mg % and [illegible]
of the proximal tibial lesion [illegible]
to prove the presence of a [illegible]

Case [illegible] Bone Tumor Registry, Radiopatologiska Institutionen The patient an infant boy was noted by his mother to have a swelling in the left inguinal region on the day of birth in May 1928 In 1931, irradiation therapy was given to the

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In these animals the fibroma was largest, while in rabbits which had received smaller doses of the prednisolone the lesion did not become larger than the ones seen *in the controls*

The known effect of tar and carcinogenic hydrocarbons on the development of the infectious fibroma might possibly be a manifestation of an inhibited antibody formation, similar to the one obtained with cortisone It is remarkable, however, that the generalized fibromatosis following intravenous administration of the fibroma virus did not become more severe in animals treated with both tar and predni solone than in those which received tar only

Prednisolone can render the infectious fibroma in rabbits more similar to a true neoplasm, but it cannot modify its nature, namely that of a temporary lesion with spontaneous regression

SUMMARY

In prednisolone-treated rabbits the reaction of the skin to intracutaneous inoculation with fibroma virus showed a slight, initial inhibition The lesions persisted for much longer periods and became often larger in the prednisolone-treated animals The lesions presented only few inflammatory cells Intravenous inoculation of prednisolone-treated rabbits with fibroma virus gave rise to generalized fibromatosis, which was not more marked in rabbits which also had been treated with tar or 9,10-dimethyl-1,2-benzanthracene

The development of antibodies against fibroma virus was delayed *in the prednisolone-treated animals*, and *the antibody* curve was *flatter* than in untreated controls The initial inhibition of the reaction of the skin and the delayed regression of the fibroma were due probably to the inhibitory effect of prednisolone on the inflammatory reaction and on antibody formation

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[Su]mmary of Reported Cases *

[F]emur	Tibia	Fibula	Hand and Wrist Bones	Foot and Ankle Bone	Sternum	Patella	Soft Tissue and Organs	Course
+	+	+	—	—	+	—	+	Died in one year Cause of death not stated
	+	+	—	+	—	—	—	Sclerosis of tibial lesion 10 years
+	—	—	—	—	—	—	—	Fractures, followed 6 years
+	+	—	—	—	—	—	+	No follow up
	—	—	—	—	—	-	+	No follow up—bony mass of skull 15 years before diagnosis
+		—	—	—	—	—	+	Died 6 years—autopsy
		-	—	—	—	—	+	Treated with X ray—relief of pain Observed for 3 years no change
+	+	+	—	—	—	—	+	Followed 3 years—still having fractures
	+		+	—	—	—	—	Lesions known for 5 years No follow up
+	+		+	—	-	—	+	Followed 11 years—stabilized
+	+			—	—	—	-	No follow up—history of previous fractures
+	+	+	—	+	-	+	+	No follow up
	+		+	—	—	—	-	No follow up—mass in tibia known to be present for 20 years
-	—		—	—	-	-	—	5 years—no progress—no recorded therapy
+	+		+	+	—	—	—	One lesion progress Followed 12 years
+	+	+			+	—	+	Lesions stabilized Followed 32 years

The lesions consisted of round defects, 0.5–1 cm wide and generally surrounded by a narrow zone of sclerosis No changes whatsoever were seen in the right femur, left humerus the hands or the feet The cervical vertebrae were not examined

Subsequent roentgenographic examinations were made repeatedly from 1940 to 1960 The extensive alterations in the pelvis vertebrae ribs and left femur were steadily regressing in the period between 1939 and 1943 and later (Figs 3, 4 and 5) Even the small lesion in the right radius gradually decreased in size (Fig 6) The small lesions in the right humerus and right tibia disappeared leaving no traces



Fig 1

Case 330 Lesion in upper end of humerus Fairly well defined rarefaction mainly in epiphysis

TABLE

	Age When Diagnosis Made	Sex	Skull	Vertebra	Pelvis	Clavicle	Scapula	Ribs	Humerus	Radius	
Parson & Ebbs	14	F	+	—	+	+	—	+	+	—	—
Ackermann & Hart	15	M	—	—	—	—	—	—	—	—	—
Jacob & Kimmelstiel	3	M	+	—	+	—	+	+	+	—	
Jacob & Kimmelstiel	11	F	+	—	+	—	+	+	+	+	+
Paden & Mantz	27	M	+	—	+	+	—	—	—	—	—
Cohen & Craig	3	F	+	+	+	+	+	+	+	—	
Czerniak & Schorr§	65	F	—	+	—	+	+	—	—	—	
Ritchie & Zeier	2	M	+	+	+	+	+	+	+	+	
Gramiak, Gilberts & Campeti	15	M	+	+	+	—	+	+	+	—	
Hayes & Brody	11	M	—	—	+	—	—	—	+	—	
Shopfner & Allen	15	M	+	—	—		-	+	+	—	
Stock (Case 4)	14	M	—	—	—		—	—	—	—	
Lie	69	M	—	—	—	—	—	—	—	+	
Pierson, Farber & Howard	16	M	+	+	+	+	+	+	+	+	
Lindbom & Spjut	27	F	—	—	—	—	—	—	+	+	
Lindbom & Spjut	3	M	+	+	+	—	+	+	+	—	

* Aegerter's case not included because of lack of details. § Hereditary telangiecto

region. The mass regressed, then again gradually increased in size, being associated with pain. In 1939 he was again treated with irradiation to lesions of the pelvic area. After treatment pain diminished. In 1943 and 1944 additional irradiation was administered to the pelvic region. The right pelvic region received in all a tissue dose of approximately 1000 r, the left 2000–3000 r. The patient also had headaches, episodes of unconsciousness and spastic paresis of both lower extremities were noted in 1939. Destructive lesions in the skull were demonstrated by roentgen examination. For these lesions the patient received irradiation therapy in 1939, 1943, 1944 and 1947, in all 1000–2000 r. Since 1947 (to 1960) the patient remained symptom free and has full range of motion of the hip joint.

In February 1939 a local resection of a lesion of the left fibula was made along with a biopsy of the soft tissues of the hip region. The former was a typical cavernous haemangioma, the latter being a lymphangioma.

Roentgenographic Findings

Case 380. Roentgenographic examination revealed lesions in the upper end of the humeri (Fig. 1), in the left head of the radius, in the proximal end of the left tibia, the left talus, distal left femur and in several of the bones of both hands. A slow but gradual progression of the lesions in the right knee were noted between 1948 and 1959 (Fig. 2). The other lesions of the skeleton had not been re-examined. In this case the skeletal changes were for the most part localized near the epiphyses, occasionally involving the metaphyses but not the diaphyses. The areas of destruction were generally well defined but not surrounded by sclerosis. A few bony trabeculae apparently remained in the areas of destruction.

Case 403. Examination in 1939 demonstrated extensive changes in nearly the entire bony pelvis (Fig. 3 a) predominantly on the left side. In addition changes were manifest in the sternum, lumbar vertebrae, ribs (Fig. 4 a) and the left femur (Fig. 5 a). Solitary lesions were noted in the skull, left fibula, right radius (Fig. 6 a) and left scapula. There were small alterations in the right humerus and right tibia

ummary of Reported Cases *

Femur	Tibia	Fibula	Hand and Wrist Bones	Feet and Ankle Bones	Sternum	Patella	Soft Tissue and Organs	Course
+	+	+	—	—	+	—	+	Died in one year Cause of death not stated
	+	+	—	+	—	—	—	Sclerosis of tibial lesion 10 years
+	—	—	—		—	—	—	Fractures followed 6 years
+	+	—	—	—	—	—	+	No follow up
		—	—	—	—	—	+	No follow up—bony mass of skull 15 years before diagnosis
+		—	—	—	—	—	+	Died 6 years—autopsy
		—	—	—	—	—	+	Treated with X ray—relief of pain
								Observed for 3 years, no change
+	+	+	—	—	—	—	+	Followed 3 years—still having fractures
	+	—	+	—	—	—	—	Lesions known for 5 years No follow up
	+		+	—	—	—	+	Followed 11 years—stabilized
	+			—	—	—	—	No follow up—history of previous fractures
	+	+	—	+	—	+	+	No follow up
	+		+	—	—	—	—	No follow up—mass in tibia known to be present for 20 years
			—	—	—		—	5 years—no progress—no recorded therapy
	+		+	+	—	—	—	One lesion progress Followed 12 years
	+	+	—	—	+	—	+	Lesions stabilized Followed 32 years

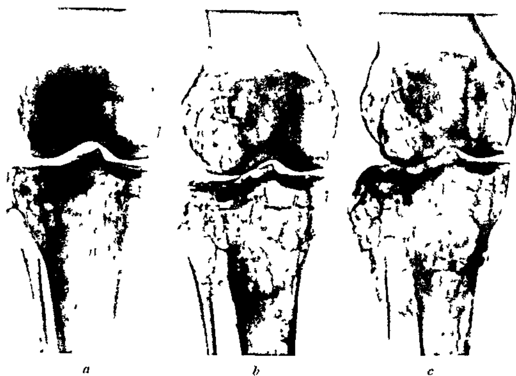
The lesions consisted of round defects 0 5–1 cm wide and generally surrounded by a narrow zone of sclerosis No changes whatsoever were seen in the right femur left humerus the hands or the feet The cervical vertebrae were not examined

Subsequent roentgenographic examinations were made repeatedly from 1940 to 1960 The extensive alterations in the pelvis vertebrae ribs and left femur were steadily regressing in the period between 1939 and 1943 and later (Figs 3 4 and 5) Even the small lesion in the right radius gradually decreased in size (Fig 6) The small lesions in the right humerus and right tibia disappeared leaving no traces



Fig 1

Case 350 Lesion in upper end of humerus Fairly well defined rarefaction mainly in epiphysis



a b c

Fig 2

Case 380 Progressive lesions in upper end of tibia and lateral femoral condyle
a) 1948 in connection with fracture of lateral condyle of tibia b) 1954 c) 1959
Slowly increasing expansion and destruction of tibia

From the beginning two lesions had been manifest in the left scapula one of which gradually disappeared without a trace The second lesion which involved the glenoid fossa remained as a defect in 1960 The lesions of the skull behaved differently from those described above The small lesion seen in the beginning of 1939 was noted to increase in size by the end of 1939 and examinations in the period between 1939 and 1943 showed the presence in the parietal region of a tiny lesion which slowly increased in size Both of these lesions progressed rapidly from 1943 to 1944 In 1947 both for the most part appeared to be filled in with bone In 1947, ence phalography and cerebral arteriography were made but no abnormal findings were noted

No new lesions have appeared since the original roentgenographic examination *in January 1939 Most of the lesions have gradually decreased in size except those* in the skull As mentioned one lesion in the left scapula has remained entirely unchanged The examination in 1960 revealed that the pelvis was severely deformed the left half being smaller than the right The structure of the left pelvis bones and the left femur (Fig 5 c) and several of the ribs is irregular (Fig 4 b) The remainder of the bone appear to be normal

Pathological Findings

The specimens from Case 380 were small biopsy fragments described as having the appearance of fat and greyish white tissue In Case 403 the fibular lesion was multi loculated fairly well defined haemorrhagic and surrounded by thin cortical bone In neither case were cystic spaces (6) described by the surgeon

Histologically the three biopsies from Case 380 were similar The first biopsy was made in 1948 the last two in 1955 *All* showed numerous small varied sized vascu lar spaces admixed with immature fibrous tissue fresh blood fibrin and foci of lymphocytes and leukocytes Most of the vessels were thin walled without detectable media They had the appearance of lymphatic channels a few contained precipitated

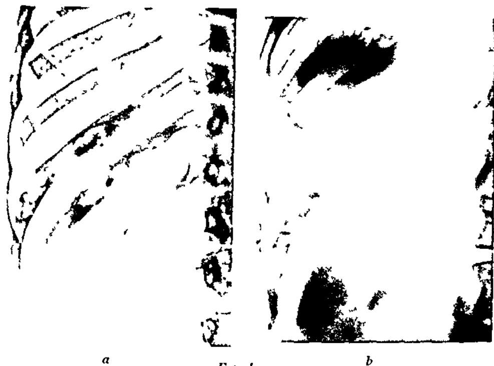


Fig 3a



Fig 3b

Ca 403 a 1934 Extensive irregular defects in greater part of pelvis and in upper part [illegible] b 1947 In right half of pelvis almost normal structure Lesions remain near left acetabulum Irradiation was given between a) and b) predominantly to the left side



a *Fig 4* *b*

Case 403 Lesions in ribs *a*) 1939 *b*) 1960 No irradiation given to this region Lesions in 10th rib disappeared almost totally in 1960 in 11th ribe some irregularities remain

protein material In some areas small veins were present The fibrous tissue was fairly vascular and often contained freshly extravasated blood The few bone fragments displayed slight, irregular erosion of a few of the bony trabeculae with scattered foci of osteoclasts In the Bone Tumor Registry this lesion was interpreted as a vascular malformation predominantly of a lymphangiomatous nature (Fig 7)

The one specimen from Case 403 showed large varied sized thin walled vessels engorged with blood The bony trabeculae demonstrated no particular change except that they seemed to have been pushed aside by the vascular lesion This lesion was interpreted as an angiomatous malformation of bone (Fig 8)

DISCUSSION

The course of this relatively rare disease has not been well outlined since few of the cases have had any lengthy follow-up Of the four cases observed for three years or more, two (3 12) showed progress of the skeletal lesions, one remained stable (1) for 10 years, in a forth patient (7) the skeletal lesions progressed and finally became stationary during the last 2½ years of an 11-year-observation period The two patients described in this paper have been followed for 12 and 32 years The courses of the two conditions have been rather deviating, the lesions in the woman having been slowly progressive no therapy had been given In the man it was noted that for a time, as a boy, certain of the lesions became larger, some gradually, others fairly rapidly By the time the patient had attained the age of 20 years several of the lesions had regressed, others had stabilized The rôle of



Fig 5

Case 403 Spontaneous disappearance of lesions in femur No irradiation given to distal part of bone *a*) 1939 *b*) 1943 *c*) 1960 Only a slight irregularity of osseous structure remains in 1960

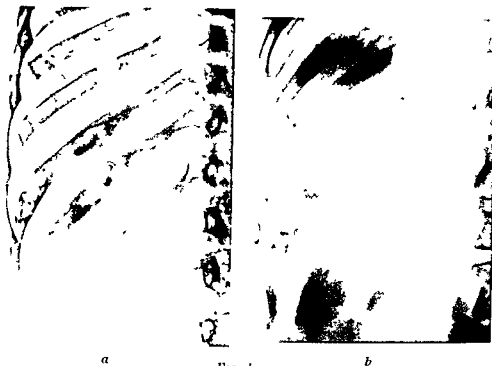


Fig 3

Case 403 Lesions in ribs *a*) 1939 *b*) 1960 No irradiation given to this region Lesions in 10th rib disappeared almost totally in 1960 in 11th ribe some irregularities remain

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a Fig 4 b

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Fig 5

Case 403 Spontaneous disappearance of lesions in femur No irradiation given to distal part of bone *a*) 1939, *b*) 1943, *c*) 1960 Only a slight irregularity of osseous structure remains in 1960



Fig 6
Case 403 Changing appearance of lesions in proximal part of radius *a*) 1939 *b*) 1943 Later these lesions disappeared

Fig 7
Case 380 Numerous thin walled variably sized vascular channels are seen Most are empty others contain protein precipitate or blood This biopsy was done in November 1948 and was from the tibia Biopsies of the tibia and little finger in 1955 were histologically similar



Fig 8

Case 403 Thin walled bl d filled vascular spaces are seen between the bony trabeculae Th s was fr m the fibula resected In 1939

irradiation therapy is difficult to assess Irradiation administered to the left half of the pelvis resulted in a regression and apparent disappearance of many of the bone lesions However a similar course was noted in bones receiving no irradiation therapy *Czerniak & Schorr* (4) reported relief of bone pain after irradiation therapy of haemangiomatous lesions Whether the bone lesions actually changed was not stated Whether a therapy is available which is adequate and efficient in cases of multiple angiomatous lesions of the skeleton is not apparent from the literature The course of the disease in one of our two patients suggests that it may be slowly progressive but also that not all of the lesions participate Some lesions may actually regress while most of the others remain stable In our second patient the process seemed to become stabilized as the patient aged Other investigators (13) have found the lesions to be progressive irrespective of therapy

Table 1 summarizes the major findings of the cases of skeletal angiomatosis Some were reported as lymphangiomas these are included because the radiographic findings are similar to the ones interpreted as haemangiomas In addition it is not uncommon for angiomatous lesions to present mixed blood vessels and lymph vessels

Clinical diagnosis of skeletal angiomatosis may often be incorrect if made prior to a biopsy of the lesion (9) This was true in our two patients, one of whom was considered to have a hyperparathyroidism and the other a Gaucher's disease The clinical misinterpretations are probably ascribable to the rare occurrence of this entity The several theories concerning the origin of angiomatosis of the skeleton have been thoroughly discussed in other papers (7 8) From the study of our cases we can add nothing new

SUMMARY

The clinical, serial roentgenographic findings and pathological observations of two cases of skeletal angiomatosis are presented Long term follow-up covering 12 and 32 years, respectively suggest that the lesions are prone to stabilize, many to regress spontaneously Irradiation may represent a remedy by which the angiomas may regress

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FROM KAPTEIN W. WILHELMSEN OG FRUES BAKTERIOLOGISKE INSTITUTT, OSLO UNIVERSITY AND RIKSHOSPITALET, OSLO (HEAD: PROFESSOR S. DICK HENRIKSEN, M.D.)

SOME OBSERVATIONS CONCERNING PHAGE-RESISTANT MUTANTS IN AN ENZYME PRODUCING PHAGE-HOST CELL SYSTEM OF E. COLI

By

SVERRE LIE

Received 9 viii 61

Sertic (1929 a) succeeded as the first to isolate a phage active against an encapsulated strain of E. coli, which produced plaques *surrounded* by a great translucent halo. Since then several workers have observed this phenomenon with phages active also against Aerobacter and *Klebsiella* species. *Humphries* (1948) working with Klebsiella pneumonia type A, fo[illegible]
the lysis [illegible]
lethal eff[illegible]
enzyme a[illegible] against a mucoid strain of Klebsiella *pneumonia, type B*, to be dependent upon the genotype of both the *bacterium* and the phage.

A susceptible host can acquire phage-resistance by *mutation or* by becoming lysogenic. Mutation may involve any *number of distinct* physiological mechanisms of which only a few are known at the moment. Lack of phage adsorption may be *due either to the* absence of receptor substance or to the covering *of the same* with some other bacterial layer, as in the mutation *from rough* to smooth in Salmonella (*Burnet* 1930). Some *mutations* are accompanied by a loss of the ability to synthetize *growth factors such* as tryptophan (*Anderson* 1946) or proline (*Wollman* 1947).

Acquisition of [illegible]
by *Burnet & Lush* [illegible]
antigenic change [illegible] of the surface of the host cell as a result of lysogenization have been reported in Salmonella (*Uetake et al* 1955).

The present paper deals with a phage active against a strain of E. coli, and some observations made on the phage-resistant mutants of this host.

Clinical diagnosis of skeletal angiomatosis may often be incorrect if made prior to a biopsy of the lesion (9) This was true in our two patients, one of whom was considered to have a hyperparathyroidism and the other a Gaucher's disease The clinical misinterpretations are probably ascribable to the rare occurrence of this entity The several theories concerning the origin of angiomatosis of the skeleton have been thoroughly discussed in other papers (7 8) From the study of our cases we can add nothing new

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Fig 1

Fig 1 Plaque morphology of phage "Balmi" Two types can rough- [illegible] ing [illegible] One can easily distinguish between "S"- and "R"-forms In the middle of the figure a smooth mutant releases phage attacking an "R" mutant

Fig 2b Phage-resistant mutants of KB 6234 61 isolated by the soft agar layer method Phage mutants produces plaques on the "R"-mutants The small dense colonies are those growing under the agar-surface



Fig 2a

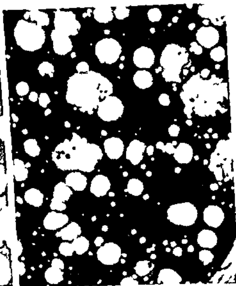


Fig 2b

2 In some of the "R"-colonies small plaques could be seen

3 Where an "S"-colony grew into contact with an "R"-colony, a clear area could sometimes be seen which surrounded the former In this area the growth of the "R"-form was greatly repressed

Phenomena 2 and 3 were best seen when the resistant mutants were selected according to the soft-agar layer method

MATERIALS AND METHODS

The phage termed "Balmi" and its host bacterium (KB 6234/61) were both isolated from a patient suffering from a severe case of cystitis. On the original plate from the urine specimen plaques surrounded by large translucent haloes were observed in the carpet of bacterial growth.

The phage could easily be isolated and propagated on its host bacterium. Care was taken in order to have a single line suspension of phage.

The host bacterium produced smooth colonies on complete medium. Glucose, lactose, maltose and mannitol were fermented with gas production, while saccharose was not fermented. Indol and methyl-red reactions were positive, while the Voges-Proskauer, citrate and urease reactions proved to be negative. The strain neither liquefied gelatin nor produced H_2S. It grew well on the "minimal medium" of *Davis & Mingioli* (1950).

"L broth" and agar (Difco) were used as solid and liquid complete media. "L broth" contained per liter: 10 g Bactotryptone, 5 g Yeast extract, 5 g NaCl, 1 g glucose; pH was adjusted to 7.0 with 1 N NaOH.

Plaque morphology was examined by means of the soft-agar layer method. The phage was propagated according to the method described by *Swanstrom & Adams* (1951).

Resistant mutants were obtained by two methods:

1. Approximately 10^8 cells were mixed with an excess of phage in 2.5 ml melted soft agar, poured on plates and incubated at 37° C for 24 hours.
2. The bacteria plus excess phage lysate were mixed and poured directly on the plate, dried and incubated ("pouring method").

RESULTS

Plaque-size and morphology. Plating of the urine specimen after centrifugation with the host strain of E. coli, revealed plaque-types which could roughly be divided into two types. This becomes apparent when studying Fig. 1. The first type had a rather small central clearing (the plaque proper) and a great halo consisting of several zones. The other type showed great central clearings (up to 5 mm in diameter) but had a rather narrow halo. The phages corresponding to the plaque-types were subcultured separately. The picture was as a whole indistinguishable from that given by a Klebsiella phage (*Park* 1956).

Host range. This was examined by plating the phage in a high titer (10^8 particles) against 25 strains of E. coli, 5 of Aerobacter and 5 of Klebsiella. None of these strains proved to be sensitive.

Phage resistance. Resistant mutants of the host bacterium showed the same picture regardless of the phage-type used for selection. Upon observation of the mutants 3 remarkable phenomena could be observed as illustrated in Figures 2 a and 2 b.

1. The resistant organisms could be divided into two groups according to their colony-morphology. One (later called the "S" form), presented smooth, raised, glistening colonies with a homogenous structure and a regular and circular margin. These colonies were quite indistinguishable from those of the susceptible strain. The other group (later called the "R" form), presented rough, flattened colonies with an irregular margin. Out of 500 colonies counted, 46 per cent were "S"-forms, 49 per cent "R"-forms, while 5 per cent could not be classified with certainty.

The phage-resistant mutants were now plated with the original sensitive strain and one of the "R"-mutants as indicators. None of the "R"-mutants proved to be lysogenized, while 32 (27.7 per cent) of the "S"-mutants liberated phage. This phage was indistinguishable from "Balmi-X", and attacked "R"-forms as well as the original strain. None of the 64 "S"-mutants investigated were found to be lysogenized with "Balmi".

These findings may reasonably explain the initial observation designated phenomenon 3, as all the "S"-forms surrounded by zones, proved to be lysogenized with "Balmi-X".

A plating of "Balmi-X" in high number against the original bacterial strain, revealed only smooth resistant colonies. Out of 32 such colonies, 13 had been lysogenized.

DISCUSSION

The studies described in the present paper indicate that resistance in the "R" mutants is due to alterations in the surface of the host cell. The colony-morphology is changed, the mutants remain sensitive to the mutant phage "Balmi-X", and no prophage-state can be detected.

Approximately 30 per cent of the "S"-mutants obtained by a selection with lysates of the original phage, apparently acquire resistance by lysogenization with "Balmi-X". How the other 70 per cent become resistant remains obscure. When taking a probable genetic lability of the *phage* into consideration, a possible explanation could be that they are all lysogenized by mutant phages, but that no sensitive indicator *strain has been found*. A plating of 20 of the non-l[illegible]

against 42 "R"-colonies

the state of lysogeny co[illegible]

assay. The system might be unable spontaneously to release phage, it might even be uninducible.

The mutant phage "Balmi-X" is capable of producing enzyme, as shown by plating it against the original *sensitive host. However*, when it produces plaques on the "R"-forms, these are not surrounded by any halo. This phenomenon may well be explained, if the receptor substance of the phage "Balmi" is *considered the* same as the substrate of the enzyme. When mutation to resistance is due to a loss of the receptor substance, the mutant *will of course* prove insensitive to the action of the enzyme. Another possible explanation might be that the mutation to resistance involves a loss of some biosynthetic faculty indispensable for the enzyme production.

SUMMARY

An enzyme-producing phage-host cell system in E. coli is described. Phage resistant mutants may *roughly be divided* into two groups according to their colony [illegible]

([illegible] smooth

rough,

Investigations were next performed in order to find some explanation to these phenomena

At first 68 "S"-forms and 42 "R"-forms were isolated and sub cultured several times in order to avoid any contamination with phage The following properties of the mutants were studied:

1 Morphology and growth in fluid medium
2 Presence of capsules
3 Stability in saline solutions
4 Nutritional requirements
5 Lysogeny

The first four investigations failed to show any differences between the two types of phage resistant bacterial mutants Growth in fluid medium gave rise to an even turbidity without sediment No tendency towards chain formation could be observed in the microscope —Capsules were demonstrated in both forms by a "serum-method". A thin smear of bacteria suspended in serum was allowed to dry, heated in a flame and stained with carbol-fuchsin (*Jyssum*, personal communication) No agglutination was caused by salt concentrations up to 8 per cent NaCl (method by *Waaler* 1935) —All the mutants investigated grew well on minimal medium

When investigating the fifth point, the existence of lysogeny, however, a remarkable difference was found between the two types of phage resistant mutants The state of lysogeny was shown to be intimately connected with the initial observations designated phenomenon 2 and 3 A single plaque which appeared in an "R"-mutant was picked and plated in soft-agar technique against the same strain The plaques thus produced differed in morphology from those produced by the original phage on its sensitive host No halo could be observed, and the plaque itself had a more turbid appearance Subsequent isolations and propagations confirmed the observation, and the phage was consequently considered a mutant and designated "Balmi-X" This phage also produced plaques on the original sensitive strain The morphology of these plaques was indistinguishable from that of 'Balmi s"

All the host cell mutants isolated were then plated with lysates of "Balmi" and "Balmi-X" "Balmi" did not produce plaques on any of these bacteria All the "R"-mutants, however, were lysed by 'Balmi-X", while the "S'-mutants proved resistant to both phages

The question of lysogeny was next taken under study A convenient and time-saving method for the detection of a spontaneous release of phage, was to spot or cross-streak the bacteria in question on a plate evenly inoculated with a fairly heavy suspension of the indicator strain After incubation for 24 hours, lysogenic strains were surrounded by a zone where no growth of the indicator strain was observed and from which great amounts of phage could be isolated

FROM KAPTEIN W WILHELMSEN OG FRUES BAKTERIOLOGISKE INSTITUTT, OSLO UNIVERSITY AND RIKSHOSPITALET, OSLO

IMMUNOCHEMICAL STUDIES ON SOME SEROLOGICAL CROSS-REACTIONS IN THE KLEBSIELLA GROUP

8 *Some Attempts to Analyse the Antigenic Specificity of Klebsiella Type 1 Polysaccharide*

By

JORUNN ERIKSEN and S D HENRIKSEN

with technical assistance of *Unni Maini*

Received 21 viii 61

Inhibition experiments and studies on the effect of oxidation with periodic acid have been utilised to great advantage in attempts to determine the chemical basis of antigenic specificity of O-antigens of the Enterobacteriaceae (3, 4) The purpose of this paper is to report similar, although less successful, experiments with a Klebsiella capsular polysaccharide

MATERIAL AND METHODS

Strains K pneumonia type 1, strains Sc and 1265

Immune sera Prepared against the same two strains The anti Sc serum had a titer of 64 in the capsular reaction and the anti-1265 a titer of 256

Inhibition experiments were carried out by the method described by *Staub & Tinelli* (3) with all of the sugar components found in the polysaccharide fucose, galactose glucose and uronic acid Since the nature of the uronic acid was unknown, both glucuronic and galacturonic acid were tested likewise [illegible]

[illegible] salt was cooled and transferred to the column without neutralisation First the column was washed through [illegible]

irregular colonies The smooth, phage-resistant bacteria are of two types Approximately 30 per cent proved to be lysogenized with a mutant phage, while in the remaining 70 per cent no evidence of lysogenization could be found None of the rough variants of the host bacterium were found to carry any prophage —The temperate mutant phage attacks the original strain with plaques surrounded by a halo It also attacks the rough bacterial variants, but in this case with no halo Some possible explanation of these phenomena are discussed

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FROM KAPTEIN W. WILHELMSEN OG FRUES BAKTERIOLOGISKE INSTITUTT, OSLO UNIVERSITY AND RIKSHOSPITALET, OSLO

IMMUNOCHEMICAL STUDIES ON SOME SEROLOGICAL CROSS-REACTIONS IN THE KLEBSIELLA GROUP

8. *Some Attempts to Analyse the Antigenic Specificity of Klebsiella Type 1 Polysaccharide*

By

JORUNN ERIKSEN and S. D. HENRIKSEN

with technical assistance of *Unni Maini*

Received 21.viii.61

Inhibition experiments and studies on the effect of oxidation with periodic acid have been utilised to great advantage in attempts to determine the chemical basis of antigenic specificity of O-antigens of the Enterobacteriaceae (3, 4). The purpose of this paper is to report similar, although less successful, experiments with a Klebsiella capsular polysaccharide.

MATERIAL AND METHODS

Stra[illegible]
Imm[illegible] serum had a titer of [illegible]
Inhib[illegible] were carried out by the method described by *Staub & Tinelli* (3) with all of the sugar components [illegible] galactose, glucose and uronic acid. Sin[illegible] both glucuronic and galacturonic acid [illegible] from type 1 polysaccharide by [illegible]

[illegible] hours with [illegible] solution [illegible] without neutralisation [illegible]

[illegible] zone adjacent to a brownish red spot [illegible] since anilin oxalate [illegible]

Oxidation of the capsular polysaccharide with periodic acid was carried out as described by *Staub & Tinelli* (3).

RESULTS

Table 1 shows the results of precipitin determinations in anti-Sc immune serum. The antigen dose which brought down a maximum of antibody, 71 μg, was chosen for the first inhibition experiments. The results are shown in Table 2. Neither fucose, nor galactose and glucose had any significant inhibitory action. The oxidised polysaccharide, presenting the same spots as before oxidation in paper chromatography, precipitated 15 per cent only of the antibody.

TABLE 1

Quantitative Precipitin Determinations in Anti Sc Serum with Klebsiella Type 1 Polysaccharide (0.5 ml Serum)

μg antigen added	20	41	71	102	122
μg antibody N pptd	83	105	116	111	111

TABLE 2

Inhibition Experiments in Anti Sc Immune Serum (0.5 ml Portions). Precipitation Tests Carried Out Later with Type 1 Polysaccharide

Antigen	Inhibitory agent added per 0.5 ml	Antibody N precipitated in μg	Per cent inhibition
Polysaccharide	Fucose, 40 mg	111	0
	Galactose 40 mg	98	12
	Glucose 40 mg	105	5
"	None	111	–
Oxidised polysaccharide	None	17	85

TABLE 3

Quantitative Precipitin Determinations in Anti 1265 Serum with Klebsiella Type 1 Polysaccharide (0.5 ml Serum Portions)

μg Antigen added	20	40	70	100	150	200	300	400
μg Antibody N pptd	161	282	401	458	486	483	488	495

Table 3 shows the results of precipitin determinations in anti 1265 serum. A dose of 100 μg antigen was chosen for inhibition experiments. Results of such experiments with this serum are presented in Table 4. None of the tested substances had any significant inhibitory action.

DISCUSSION

Methods successfully used in studies on the specificity of O-antigens do not seem to work equally well with Klebsiella capsular polysaccharides. None of the componens present in the polysaccharide, whether isolated or combined, nor a uronic acid isolated from the polysaccharide, not even a neutralised hydrolysate, had any inhibitory effect on the reaction between the polysaccharide and the homologous immune serum.

TABLE 4

Inhibition Experiments in Anti 1265 Serum (0 5 ml Portions)
Precipitation Tests Carried Out Later with Type 1 Polysaccharide

Inhibitory agent added per 0 5 ml serum	Antibody N precipitated in µg	Per cent inhibition	pH in supernate after pptn
40 mg galactose	440	2 7	7 8
40 mg fucose	452	0	7 7
40 mg glucose	436	3.5	7 7
50 mg neutralised galacturonic acid (pH 6 6)	419	7.3	7 7
40 mg neutralised glucuronic acid (pH 6 7)	435	3 8	7 5
42 mg uronic acid fraction from column chromatography (pH 6 5)	423	6 4	6 7
Glucose + galactose + Fucose in same proportions as in 30 mg hydrolysate of polysaccharide 1265	422	6 6	7 8
Neutralised hydrolysate of 30 mg polysaccharide 1265	437	3 3	7 7
Neutralised hydrolysate of 30 mg polysaccharide 1265 hydrolysed with 0 5 N H_2SO_4	432	4 4	7 6
None	452	–	7 9

A certain hesitation must be displayed before conclusions are drawn from negative results but it might be suggested that the determinant groupings in the Klebsiella capsular polysaccharides must differ from those of the O antigens Other methods must be introduced, apparently if any insight is to be gained into the nature of the determinant structures of klebsiella polysaccharides

The fact that oxidation with periodic acid greatly reduces the serological reactivity of the polysaccharide, indicates that some of the components playing a part in the determination of specificity, are destroyed but since none of the spots in the chromatogram disappeared, the nature of these components remain unknown

SUMMARY

Inhibition experiments and tests with oxidised capsular polysaccharide of Klebsiella type 1 failed to give any useful information about the chemical basis of the antigenic specificity

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5

FROM KAPTEIN W. WILHELMSEN OG FRUES BAKTERIOLOGISKE INSTITUTT, OSLO UNIVERSITY AND RIKSHOSPITALET, OSLO

ATTEMPTS TO DEMONSTRATE AUTOANTIBODIES IN COLITIS ULCEROSA

By

S. D. HENRIKSEN, WENCHE BLIX GUNDERSEN
and ROALD OPSAHL JR.

Received 11. x. 61

In 1958 *Broberger & Perlmann* (4) examined a group of children suffering from colitis ulcerosa and demonstrated, in the majority of the cases a presence in the serum of antibodies reacting with extracts of colonic mucous membrane. In a subsequent paper *Asherson & Broberger* (1) showed that haemagglutinating antibodies against erythrocytes sensitized with colonic extracts could be demonstrated in the sera of 85 per cent of 14 children, but only in the sera of 38 per cent of 36 adults suffering from ulcerative colitis. Antibodies were also found in some cases of disseminated lupus erythromatosus, parenchymal liver disease, rheumatoid arthritis and nephrosis. According to *Broberger* (3) active extracts were obtained only from tissues of infants who had died without feeding during their first day of life, as described by *Broberger & Perlmann* (4).

Polcak & Vokurka (6) prepared saline extracts of colonic mucous membrane and the submucosa and used these to sensitize collodion particles. With such antigens 100 per cent positive results were obtained in 30 colitis sera and 100 per cent negative results in 175 sera from patients suffering from other diseases (including lupus erythematosus disseminatus) and in 20 normal sera.

Bregman & Kirsner (2) also reported the demonstration of autoantibodies reacting with antigens prepared from colonic tissues in ulcerative colitis.

Gray, Walker & Thompson (5) prepared extracts from 12 different colons from foetuses or from adults suffering from carcinoma or ulcerative colitis.

Both saline extraction and the phenol-water extraction method, which yielded the best results in the experiments of *Broberger & Perlmann*, (4) were used. Tests with sera from 50 patients with ulcerative

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Our thanks ... friendly advice and help and to heads of departmen... artments of surgery, internal medicine, obstetri... as well as to Dr. *Olav Refvem* for kind help in

colitis with an age distribution between 16 and 57 years by the gel diffusion or haemagglutination techniques gave entirely negative results

MATERIAL AND METHODS

Extracts were prepared of colonic mucous membranes from newly born infants still born foetuses as well as from 5 colons removed from patients suffering from ulcerative colitis In these latter cases the abundance of scar tissue made an accurate separation of the mucous membrane from the underlying tissues impossible but as much as possible of the external layers was removed Both saline extraction and the phenol water extraction method described by *Broberger & Perlmann* (4) were used Experiments were performed with the fraction precipitated from the water phase by 1 volume of ethanol and with the fraction precipitated with 10 volumes of ethanol as well as with a fraction which could be eliminated from the phenol phase by addition of ethyl ether Some extracts of kidney tissue were also prepared

The serological methods used included indirect haemagglutination with sensitized human group O erythrocytes or sheep erythrocytes in the latter case after absorption of heterophile antibodies with unsensitized cells further agglutination of sensitized achrylate particles complement fixation and gel diffusion by a method described by *Ouchterlony*

Altogether 33 sera from patients suffering from ulcerative colitis were examined among which no less than 8 were from children between 2 and 14 years of age (ages were unknown in some of the cases) A positive control serum received from Dr *Broberger* was used for some of the tests In 4 cases sera taken from patients with ulcerative colitis before colectomy were tested against extracts of the patients' own colonic tissue

In addition some experiments were made in order to produce if possible auto immune disease in guinea pigs by injecting subcutaneously into young guinea pigs a mixture of finely divided suspensions of foetal tissue and Freund s adjuvants

RESULTS

Not a single positive results was obtained

DISCUSSION

Our results, which are in fair accord with the ones obtained by *Gray, Walker & Thompson* (5), fail to explain the discrepancy with previous positive findings Considering the results obtained by *Polcak & Vokurka* (6) showing a one hundred per cent reliable distinction between sera from colitis patients on one hand and sera from a wide variety of other diseases and from healthy individuals on the other, it is remarkable that experiments with the very similar, although not quite identical technique here employed, were to give entirely negative results

The discrepancy with the results published by *Broberger & Perlmann* (4) and by *Asherson & Broberger* (1) need not be quite as marked as here indicated *Broberger* states (3) that colonic extracts give variable results and also that the majority of extracts is inactive Furthermore, it appears to be of decisive importance to take the tissues from newly born infants and to have the tissues extracted within one hour after death With a few exceptions we never succeeded in obtaining such tissues a feature which may explain our negative findings Besides our methods may have differed in significant details from the ones de-

scribed by *Broberger & Perlmann* (4), although we are unaware of such difference

Certainly additional research is required in order to clarify the origin and significance of the discrepancies and to determine the rôle of auto immune phenomena in ulcerative colitis

SUMMARY

Attempts to demonstrate antibodies reacting with extracts of colonic tissues in sera from patients suffering from ulcerative colitis failed to give positive results

REFERENCES

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THE LABORATORY CONTROL OF HOSPITAL STAPHYLOCOCCAL INFECTIONS

By

B. J. TORHEIM

Received 3.viii.61

Several reports have appeared during the last years concerning staphylococci occurring in hospitals and the problems arising in connection with staphylococcal infections (*Rountree & Barbour* 1951, *Vogelsang* 1953, *Blair & Carr* 1958, *Koch, Kastensen & Resnick* 1959, *Bass & Felton* 1959, *Finland, Hirsch & Wallmark* 1960, *Vogelsang & Haaland* 1959).

The object of the report here presented is to give an outline of some methods suitable for the continous control of occurrences of staphylococci in a larger general hospital and to submit some of the results of the work carried out during one year.

During the winter of 1959 an outbreak of staphylococcal infections occurred in one of the surgical wards of the hospital. A committee was appointed consisting of representatives for the different clinical departments and the bacteriological laboraty with a view to lay down rules to be observed in hospital infections. One representative for each department was also appointed entrusted with the task of reporting to the bacteriological laboratory all cases of staphylococcal infections occurring in his department. This representative was supposed to act as "contact man" between the department and the laboratory in questions concerning hospital infections. The committee had regular meetings where reports were discussed from the contact men in the different clinical departments and where the actual practical undertakings necessary to control the staphylococcal situation at the hospital were suggested.

MATERIALS AND METHODS

Strains. 752 coagulase positive strains were [illegible] —
les [illegible]
417
m st [illegible]
ren s [illegible]
numb [illegible]
lated
mod [illegible]

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MATERIALS AND METHODS

Strains. 752 [illegible] from patients chiefly from [illegible]

(*Chapman* 1945), after 36 hours growth at 37° C. The strains were tested for coagulase, and only coagulase positive strains were phage typed.

Phage typing. The phage typing was performed with the following set of phages:

Phages	*Group*
29, 52, 52A, 79, 80, KS6, 81	I
3A, 3B, 3C, 55, 71	II
6, 7, 42E, 47, 53, 54, 73, 75, 77	III
42D	IV
187	V

Phage 81 was obtained with its propagating strain from Professor *Th. Vogelsang*, Gades Institutt, Bergen, the rest of the phages were supplied with their propagating strains [illegible] Stockholm [illegible] wing weak [illegible] were also tested with the same phages at 1000 × RTD. Phage patterns showing overlapping between phage groups were collected in a group called M (miscellaneous). The strains showing no lysis or very weak lysis when tested with 1000 × RTD were collected in a group called NT (not typable). The readings were made in the following way:

+++	complete lysis	
++	strong lysis	more than 50 plaques
+	moderate lysis	20–50 plaques
+−	weak lysis	less than 20 plaques

Propagation and typing were carried out according to the methods outlined by *Williams & Rippon* (1952). The application of the phages on the plates was made partly according to the phage typing technique described in *Williams & Rippon* (1952), partly by means of an apparatus for phage typing (*Torheim* 1960).

Antibiotic sensitivity testing. A disc plate method was used for antibiotic susceptibility testing. The following six antibiotics were used: penicillin, streptomycin, *chloramphenicol, tetracycline, erythromycin and oleandomycin, using sensitivity* discs manufactured by Bakteriologiska Laboratoriet, Karolinska sjukhuset, Stockholm (*Ericsson, Högmann & Wickman* 1954, *Tunevall & Ericsson* 1954).

Blood agar plates containing 5 per cent horse blood were inoculated from the staphylococcal cultures used for testing.

The strains were divided into a sensitive group, including both highly and moderately sensitive strains, and a resistant group, including relatively resistant and completely resistant strains, the lower limits of the sensitive group being the following zone diameters:

Penicillin	21 mm
Streptomycin	15 mm
Chloramphenicol	15 mm
Tetracycline	16 mm
Erythromycin	19 mm
Oleandomycin	20 mm

Screening of the strains. A punch card system for the screening of the staphylococci phage typed was used. From this it was possible to pick out phage types, phage groups, type combinations most commonly occurring, RTD results and 1000 × RTD results, antibiotic sensitivity test results, patients and personnel, their names, departments of the hospital, source of isolation, diagnosis, admittance to the department, and other informations about the person from whom the staphylococcus was isolated.

RESULTS

Approximately 70 per cent of the strains from personnel and approximately 75 per cent of the strains from patients were lysed by RTD.

Table 1 shows the distribution of the strains according to source of isolation from patients and personnel.

TABLE 1

Distribution of Strains According to Source of Isolation

	No of strains from patients	No of strains from personnel
Pus	468	19
Nose	152	260
Throat	22	138
Expectorate	24	
Urine	64	
Faeces	6	
Blood culture	16	

From this table it is seen that most of the strains were isolated from pus

Table 2 shows the distribution of the 752 strains isolated from patients and the 417 strains isolated from personnel according to the different phage groups

TABLE 2

Distribution of Strains from Patients and Personnel According to the Different Phage Groups

	I	II	III	IV	V	M	NT
Number of strains from patients	351	35	144	5	25	98	94
Percentage	46.7	4.7	19.1	0.7	3.3	13.0	12.5
Number of strains from patients	157	26	73	1	9	84	67
Percentage	37.6	6.2	17.5	0.2	2.2	20.1	16.1

M miscellaneous NT not typable

Total number of strains 752 (patients) Total number of strains 417 (personnel)



Fig 1

Distribution of 752 strains of Staphylococcus aureus isolated from patients on different phage groups

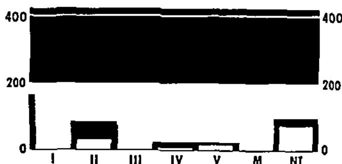


Fig 2
Distribution of 417 strains of Staphylococcus aureus isolated from personnel, on different phage groups

Fig 1 and Fig 2 illustrate Table 2, showing group I as the dominant phage group both among the strains isolated from patients and among the strains isolated from personnel

TABLE 3
Distribution of Strains within Group I According to Different Phage Types

	80/KSC/81	52/52A/79 80/KSC/81	29	Other patterns
Number of strains from patients	194	56	16	85
Number of strains from personnel	75	24	10	48

TABLE 4
Distribution of Phage Type 80/KSC/81 According to Source of Isolation

Source of isolation	No of strains from patients
Pus	158
Nose	11
Throat	1
Expectorate	8
Urine	7
Faeces	1
Blood culture	7

TABLE 5
Distribution of Phage Type 80/KSC/81 According to Diagnosis

Diagnosis	No of strains from patients
Abscesses on skin	70
Infected surgical wounds	51
Otitis	7
Conjunctivitis	2
Sepsis	8
Pneumonia	8
No probable relation to diagnosis	47

Table 3 shows the distribution of strains belonging to group 1 according to different phage types within group 1

Table 3 indicates phage type 80 KS6 81 as the most frequently occurring type Table 4 shows the source of isolation of this type and Table 5 the diagnosis

Phage type 80 KS6 81 caused mostly severe infections, in one case the patient died from sepsis, and phage type 80 KS6 81 could be isolated from the blood culture

Fig 3 and Fig 4 show the number of resistant strains to six different antibiotics within each phage group and for phage type 80 KS6 81 Fig 3 illustrates the conditions for the strains isolated from patients, Fig 4 conditions for strains isolated from personnel

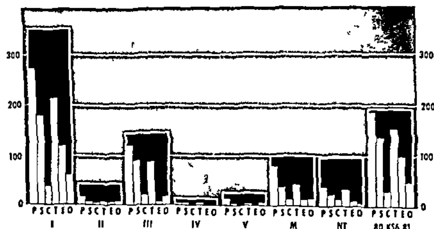


Fig 3

Distribution of resistant strains isolated from patients to six different antibiotics within each phage group and for phage type 80 KS6/81

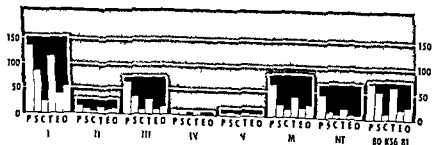


Fig 4

Distribution of resistant strains isolated from personnel to six different antibiotics within each phage group and for phage type 80/KS6 81

Black columns indicate number of strains resistant to
P penicillin S—streptomycin C—chloramphenicol T—tetracyclin
E erythromycin O—oleandomycin M—miscellaneous, NT—not typable

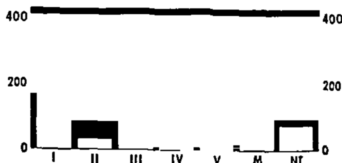


Fig 2
Distribution of 417 strains of Staphylococcus aureus isolated from personnel, on different phage groups

Fig 1 and Fig 2 illustrate Table 2, showing group I as the dominant phage group both among the strains isolated from patients and among the strains isolated from personnel

TABLE 3
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	80 KS6 81	52 52A 79 80 KS6 81	29	Other patterns
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Sepsis	8
Pneumonia	8
No probable relation to diagnosis	47

The most commonly occurring phage patterns in group I, besides type 80 KS6 81, were types 52 52A/79 80 KS6 81 and type 29

Type 52 52A/79 80 KS6 81 occurred fairly often, not infrequently these strains were isolated from persons who previously had been or later became carriers of phage type 80 KS6 81 *Rosenblum* and *Jackson* (1960) mention lysogenic conversion of staphylococcal strains of type 80 81 and related phage types

Phage patterns often occurring in group II were 3A/3B 3C/55 71, 55, 3A, 3B 71 and 3C/55 71

The most frequently occurring phage patterns in group III were 6 7/47/54 75 77, 6 7/42E/53 54/75/77, 6 47 and 7/42E 47/53 54/70 77 Overlapping between phage groups was most common between group I and III

Members of the staff, picked out as carriers of the dominating phage type, were instructed about the situation, the importance of positive cooperation was explained to them, the significance of a meticulous personal hygiene being stressed They were told to contact the outpatient department of the oto-laryngeal department for examination and treatment, if required, not until tests on 3 consecutive occasions had been negative would they be considered as non-carriers

Detailed instructions were given as to the importance of a maintenance of strict rules for personal hygiene, *e g* never to touch their nose with their fingers during their work with the patients, to clean their hands after having used a handkerchief, etc to prevent the dissemination of infective agents, to exercise caution in the treatment of infected bandages from patients, to use masks correctly, whenever required, always to be on the alert whenever furuncles and other skin infections occurred and to contact the head of the department if such infections occurred, the head of the department should be responsible for treatment to be instituted, if required, and decide whether leave of absence seemed necessary

Last not least, positive information about the importance of a careful personal hygiene has been given to the staff through lectures and by way of the daily influence in the departments This active information service together with the perpetual activation of the personnel instilled by the control carried out by the bacteriological laboratory, the committee, and the contact men in the different departments have combined to be a great help in making the personnel positive in their attitude to and their cooperation in the routine examinations, and to realize the importance of a meticulous personal hygiene in our common struggle against staphylococcal infections in the hospital

SUMMARY

Experiences from work carried out in a larger, general hospital for the purpose of keeping hospital infections, especially staphylococcal infections, under control are submitted

Figs 3 and 4 show that phage type 80 KS6 81 are more resistant to antibiotics than the strains from the phage groups I II III IV V M and VI Strains from phage type 80 KS6 81 are incluoded in group I The results for the strains isolated from patients are in accordance with the results isolated from personnel

DISCUSSION

In order to prevent occurrence of severe staphylococcal epidemics in the hospital it was necessary to accomplish a routine control of the staphylococcal infections occurring at the different departments Phage typing was therefore carried out of all coagulase positive staphylococci sent to the laboratory originating from patients for the time being in the different departments The phage typing required a great deal of extra work for the laboratory but the construction of a phage type apparatus (Torheim 1960) facilitated essentially the work By means of a punch card system it was easy to check the staphylococcal situation in the different departments

On the basis of information obtained of staphylococci isolated from patients the routine examination of personnel in the actual departments could be brought about Specimens were taken from noses and throats of the personnel in these departments in order to pick out the carriers of such staphylococcus type as was predominating in infections among patients in the departments

Figs 1 and 2 show that phage group I predominates in our results In many hospital reports from other countries (*Blair & Carr* 1953 *Knight & Holzer* 1954 *Rountree* 1953) group III has been found to have prevalence *Ortel* (1958) and *Pohn* (1957) report that group I predominate in hospital infections *Elek* (1959) says in his book about staphylococci that group I strains apparently are rare in Scandinavian countries *Vogelsang* (1959) found group III to predominate in patients and in personnel in hospitals in Bergen

Type 80 KS6 81 was the phage type of prevalence as shown in *Table 3* *In spite of the fact that this type has caused the most severe* hospital staphylococcal epidemics elsewhere the situation in this hospital so far gives no cause for alarm

Agreement is striking of results obtained with strains isolated from patients and strains isolated from personnel cf Figs 1 2 3 and 4 This agreement is seen in the occurrence of phage groups and types and also in the antibiograms *This fact emphasizes the correctness* of the attitude taken up by the committee viz to use the staphylococcal pattern found among the patients as basis for their procedures among the personnel

Figs 3 and 4 also show a high degree of resistance against antibiotics of the strains even a high degree of multiple resistance Type 80 KS6 81 showed a higher degree of resistance than strains belonging *to other phage groups*

KOMMUNEHOSPITALET, VII DEPARTMENT (CHIEF TAGE BJERING M D) AND STATENS SERUMINSTITUT ANTIBIOTIC DEPARTMENT, COPENHAGEN DENMARK

THE CONCENTRATION OF PENICILLIN IN BLOOD, URINE AND SPUTUM AFTER INJECTION OF MILLION-UNIT DOSES OF G-PENICILLIN (NATRIUM BENZYL PENICILLIN)

By

S Faurschou Jensen, Erna Lund & Inger Louise Marner

Received 3 xi 61

During recent years the therapeutical doses of G-penicillin have been increased in the hope of combating infections with bacteria less sensitive to penicillin *The reports concerning the concentration of penicillin* in blood, tissue, and urine after administration of large doses of penicillin are only scanty In the present study such concentrations have been measured after intramuscular administrations of 5 million, 10 million or 20 million units of G penicillin The work includes bacteriological examination of sputum and urine before and a varying number of hours after injection of penicillin

MATERIAL

Th [illegible] All an [illegible] contents of penicillin inhibiting [illegible]

[illegible] was examined one [illegible] was made immediately before [illegible] first injection [illegible] their ability to [illegible]

TECHNIQUE

Determination of penicillin concentration is carried out by the cup method (*K A Jensen K O Møller & K Overgaard* 1945) (*Lund* 1955) All determinations are repeated on two successive days

The standard strain of Staphylococcus aureus 209 P (F D A) (standard 8 (Wellcome) diluted 1 100) is poured on to large blood plates diameter 14 cm The neces

752 strains of coagulase positive staphylococci isolated from patients and 417 strains isolated from personnel have been phage typed and the antibiotic sensitivity has been tested during one year in which a control work was carried out Phage group I predominated in strains isolated both from patients and personnel, within group I, 80 KS6 81 was the phage type of prevalence

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sary number of holes 8 mm in diameter, are bored out and filled with the specimen or penicillin standard The plates are incubated at 37° C for 18 20 hours The diameter of the zone of inhibition is measured and read by aid of a standard curve Two of the holes in each plate are filled with varying concentrations of the penicillin standard

Two concentrations of serum are examined either undiluted and 1 10 or 1 10 and 1 100 according to the anticipated concentration Both the serum specimens and the penicillin standard used are diluted with normal human serum When measuring urine both specimen and penicillin standard are diluted with distilled water When sputum is examined the specimen is tested undiluted whereas penicillin standard is diluted with distilled water Urine collected before treatment is examined undiluted and specimens taken after treatment are examined in three dilutions 1 100 1 1000 and 1 10 000 or 1 000 1 10 000 and 1 100 000 Sputum is homogenized before determination by being drawn repeatedly in and out of a Pasteur pipette

Considerable errors are involved in determinations of the concentration of penicillin in sputum It is not certain whether the material is actually sputum or whether varying amounts of throat or mouth secretion are mixed with it although the specimens are obtained under supervision Varying amounts of penicillin fixing substances may be present in sputum

Bacteriological Examination

Urine is cultured on 5 per cent blood agar Conradi Drigalski agar Tellur medium and in 5 per cent ox serum broth Sputum is inoculated on 5 per cent blood agar Levinthal agar and into 5 per cent ox serum broth The fluid cultures are spread on solid media Determination of penicillinase formation and penicillin sensitivity is carried out on pure cultures

Determination of Penicillinase (Bondi & Dietz 1944)

Each of the bacterial strains isolated is inoculated into broth (pneumococcus and streptococcus strains into serum broth) and incubated for 18–20 hours The culture is mixed [illegible]
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+++ [illegible] per ml of G penicillin 500

++ [illegible] ml of G penicillin 50 and

\+ [illegible] G penicillin 5 50 and 500 [illegible] units per ml unchanged

(+) Reduced inhibition zone with 0 5 units per ml of G penicillin 5 50 and 500 units per ml unchanged

0 Unchanged inhibition zone with 0 5 5 50 and 500 units per ml of G penicillin

Unchanged Gives the same inhibition zone as the corresponding G penicillin standard

Penicillin Sensitivity

The strains isolated are examined as regards their sensitivity to G penicillin by means of the tablet method (*Lund* 1957) Tablets containing 25 units and 1000 units

TABLE 1

Review of the 14 Patients

No.	Sex	Age	Clinical diagnosis	Temp. on admin. Celcius degrees	S.R.	Serum creatinine in mg%	G-penicillin i.m.
1	M	62	Bronchopneum. bilat. Mb. cord. arteriosel.	38.5 died 12 hours after admin.			5 mill. U × 1
2	F	56	Bronchitis chr. Bronchiectasia bilat. Emphysema pulm.	37.8	45	1.1	5 mill. U × 2
3	M	62	Bronchitis chr. Asthma bronch.	39.4	10	1.2	5 mill. U × 2
4	M	72	Bronchitis chr. Emphysema pulm. Mb. cord. arteriosel.	39.4	20	1.2	5 mill. U × 2
5	M	36	Pneumonia crouposa dxt.	38.6	64	–	5 mill. U × 2
6	M	78	Bronchitis chr. Bronchiectasia bilat. Hypertrofia prostatae cum retentio urinae ac.	37.5	43	1.4	5 mill. U × 2
7	M	57	Leucosis aleucaemica	37.1	113	0.9	10 mill. U × 2
8	M	35	Glomerulonephritis chr. *Sinuitis max. bilat.*	37.2	64	5.5	10 mill. U × 2
9	M	54	Bronchitis chr. Bronchiectasia pulm. bilat. Emphysema pulm. Cor pulmonale chr.	37.0	1	1.5	10 mill. U × 2
10	M	61	Bronchitis chr. Bronchiectasia pulm. bilat. Emphysema pulm.	36.9	7	0.7	10 mill. U × 2
11	M	63	Bronchitis chr. Bronchiectasia pulm. bilat. Emphysema pulm.	38.0	6	0.7	10 mill. U × 2
12	F	56	Pneumonia crouposa dxt.	39.7	99	1.0	20 mill. U × 2
13	M	73	Bronchitis chr. Bronchiectasia pulm. bilat. Emphysema pulm.	37.2	32	1.2	20 mill. U × 2
14	F	73	Bronchitis chr. Mb. cord. arteriosel.	38.1	47	1.1	20 mill. U × 2

sary number of holes 8 mm in diameter are bored out and filled with the specimen or penicillin standard The plates are incubated at 37° C for 18 20 hours The diameter of the zone of inhibition is measured and read by aid of a standard curve Two of the holes in each plate are filled with varying concentrations of the penicillin standard

Two concentrations of serum are examined either undiluted and 1 10 or 1 10 and 1 100 according to the anticipated concentration Both the serum specimens and the penicillin standard used are diluted with normal human serum When measuring urine both specimen and penicillin standard are diluted with distilled water When sputum is examined the specimen is tested undiluted whereas penicillin standard is diluted with distilled water Urine collected before treatment is examined undiluted and specimens taken after treatment are examined in three dilutions 1 100 1 1000 and 1 10 000 or 1 000 1 10 000 and 1 100 000 Sputum is homogenized before determination by being drawn repeatedly in and out of a Pasteur pipette

Considerable errors are involved in determinations of the concentration of penicillin in sputum It is not certain whether the material is actually sputum or whether varying amounts of throat or mouth secretion are mixed with it although the specimens are obtained under supervision Varying amounts of penicillin fixing substances may be present in sputum

Bacteriological Examination

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Determination of Penicillinase (Bondi & Dietz 1944)

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(+) Reduced inhibition zone with 0 5 units per ml of G penicillin 5 50 and 500 units per ml unchanged

0 Unchanged inhibition zone with 0 5 5 50 and 500 units per ml of G penicillin

"Unchanged" Gives the same inhibition zone as the corresponding G penicillin standard

Penicillin Sensitivity

of G penicillin are used[1] The tablet containing 25 units gives a measure of the *in vivo* sensitivity of the strain after administration of 300 000 to 400 000 units while the tablet containing 1000 units indicates the sensitivity during treatment with million unit doses of G-penicillin

TABLE 2

Concentration of G-Penicillin in Blood Urine and Sputum after i m Injection of 5, 10 or 20 Million Units

Dose of G pen in units	Pt No	Blood Hrs after G pen			Urine Hrs after G pen			Sputum Hrs after G-pen		
		1	6-8	12 16	1	6-8	12 16	1	6-8	12 16
5 *mill* × 2	1	*30*			*10 000*					
5 mill × 2	2	60 80	4	0 1	10 000 10,000	>10,000	900	2 2 0 5	2	0 2
5 mill × 2	3	60 45	2	0 03	1,000 500		300	1 0 4	0 3	0 1
5 mill × 2	4	60 60	12	0 5	10 000 20 000	5 000	2 500	6 6	1 5	0 5
5 mill × 2	5	30 40		<0 03	7 000 9 000		1,000			
5 mill × 2	6	130 100	20	0 6	5 <0 03	20	<0 03	0 5 1 3		
10 mill × 2	7	240 210	30	2	30 000 80 000	30 000	15 000			
10 mill × 2	8	400 400		17 15	20 000 70 000		5 000			
10 mill × 2	9	100			30 000			6		
10 mill × 2	10	100 125	2	0 3	10 000	12 000	30 000			
10 mill × 2	11	110 130		1 <0 03	35 000 70 000		5 000 900	1 2 1 3		1 0 0 1
20 mill × 2	12	110 120		11 13	4 000 35 000		50 000 20 000	2 1		3 7
20 mill × 2	13	180 100		8 5 9	20 000 30 000		60 000 40 000	3 2 4 0		1 0 1 5
20 mill × 2	14	500		4	100 000		20 000			

Figures indicate the concentration of penicillin U/ml

EXPERIMENTAL

The concentrations of G-penicillin in blood, urine and sputum after intramuscular injection of 5, 10 or 20 million units are shown in Table 2 None of the specimens caused inhibition of the standard strain Staphylococcus areus 209 P before treatment Where double values are given, the upper figure denotes the concentration one hour after the first injection and the lower the concentration one hour after the second injection

[1] The tablets are kindly supplied by *R.M.C. Roskilde*

One hour after injection of 5 10 or 20 million units of G penicillin intramuscularly, a penicillin concentration of 30 to 500 units per ml could be measured in *serum* and at the same time a concentration ranging from an unmeasurable amount up to 100 000 units per ml in *urine*. Six to eight hours later the concentration had decreased considerably, both in serum and in urine, and after 12 to 16 hours the serum generally contained only very small concentrations, urine contained rather high quantities.

Two patients (Nos 6 and 8) presented higher values in serum than the other patients who received the same dose probably because of a more or less reduced renal function (serum creatinine 1 4 and 5 5 mg per cent respectively).

TABLE 3

Bacterial Strains in Urine before and after i m Injection of G Penicillin

Pt No	Dose of G-pen in units	Strains in urine			
		Before inj of penicillin	1 hour after 1st injection	Just before 2nd injection	1 hour after 2nd injection
3	5 mill × 2	Coli Klebsiella (few)	Coli Klebsiella (few)	sterile	sterile
6	5 mill × 2	Klebsiella Strept faec	Klebsiella Strept faec	Klebsiella Strept faec	Klebsiella Strept faec
9	10 mill × 2	Coli Klebsiella Strept faec Gram neg non fermenting rod Proteus	Coli Klebsiella Strept faec Gram neg, non fermenting rod	–	–
10	10 mill × 2	Gram neg non fermenting rod Strept faec (few)	–	sterile	sterile
12	20 mill × 2	Coli (few)	Coli (few)	sterile	sterile
13	20 mill × 2	Coli Gram neg non fermenting rod	Coli Gram neg non fermenting rod	sterile	sterile
14	20 mill × 2	Coli	Coli	sterile	sterile

It is seen from Table 2 that the injected penicillin is excreted rapidly in large amounts with the urine. The diureses of the 14 patients ranged from 700 to 1700 (average 950) ml during the 24 hours following the first penicillin injection. Patient No 6 had very small amounts of penicillin in the urine probably because of penicillinase producing bacteria (Klebsiella) (Table 3 and 5).

One hour after injection of 5 10 or 20 million units of G penicillin, a penicillin concentration of from 0 4 to 6 units per ml could be demonstrated in *sputum*. After 6–8 hours the values were 0 3 to 2 units

of G penicillin are used[1] The tablet containing 25 units gives a measure of the *in vivo* sensitivity of the strain after administration of 300 000 to 400 000 units while the tablet containing 1000 units indicates the sensitivity during treatment with million unit doses of G penicillin

TABLE 2

Concentration of G Penicillin in Blood Urine and Sputum after i m Injection of 5 10 or 20 Million Units

Dose of G pen in units	Pt No	Blood Hrs after G pen			Urine Hrs after G pen			Sputum Hrs after G-pen		
		1	6 8	12 16	1	6-8	12 16	1	6-8	12 16
5 mill × 2	1	30			10 000					
5 mill × 2	2	*60* 80	*4*	*0 1*	*10 000* 10 000	*>10 000*	*900*	2 2 0 5	2	0 2
5 mill × 2	3	60 45	2	0 03	1 000 500		300	1 0 4	0 3	0 1
5 mill × 2	4	60 60	12	0 5	10 000 20 000	5 000	2 500	6 6	1 5	0 5
5 mill × 2	5	30 40		<0 03	7 000 9 000		1 000			
5 mill × 2	6	130 100	20	0 6	5 <0 03	20	<0 03	0 5 1 3		
10 mill × 2	7	240 210	30	*2*	*30 000* 80 000	*30 000*	*15 000*			
10 mill × 2	8	400 400		17 15	20 000 70 000		5 000			
10 mill × 2	9	100			30 000			6		
10 mill × 2	10	100 125	2	0 3	10 000	12 000	30 000			
10 mill × 2	11	110 130		1 <0 03	35 000 70 000		5 000 900	1 2 1 3		1 0 0 1
20 mill × 2	12	110 120		11 *13*	4 000 *35 000*		50 000 *20 000*	2 1		3 7
20 mill × 2	13	180 100		8 5 9	20 000 30 000		60 000 40 000	3 2 4 0		1 0 1 5
20 mill × 2	14	500		4	100 000		20 000			

Figures indicate the concentration of penicillin U/ml

EXPERIMENTAL

The concentrations of G penicillin in blood urine and sputum after intramuscular injection of 5 10 or 20 million units are shown in Table 2 None of the specimens caused inhibition of the standard strain Staphylococcus aureus 209 P before treatment Where double values are given the upper figure denotes the concentration one hour after the first injection and the lower the concentration one hour after the second injection

[1] *The tablets are kindly supplied by R M C Roskilde*

per ml and after 12 16 hours measurable quantities were still present the highest concentration being found after injection of 20 million units of G penicillin

On the whole the content in sputum seems to parallel the content in serum but the sputum concentration is approximately 40 fold lower It may be mentioned that in previous experiments the penicillin concentrations found in tonsillar tissue were about 1 10 of the corresponding serum concentrations (*Lund & Fverberg* 1952)

Bacteria in urine (Table 3) In 7 of the 14 patients bacteria could be demonstrated in the urine before injection of penicillin in three of the patients (Nos 3 10 and 12) the numbers of bacteria were small Coli were demonstrated in four patients bacteria of the Coli Klebsiella group in three Streptococcus faecalis in three Gram negative non fermenting rods in three and Proteus in one

It will be seen that in patient No 9 the Proteus bacteria had disappeared from the urine one hour after the first injection of penicillin The urine taken immediately before the second injection had become sterile in patients Nos 3 10 12 13 and 14 Patient No 9 was observed only until after the first administration of penicillin Thus just before the second penicillin injection only one of the six patients examined (No 6) had bacteria in the urine *vi* Klebsiella and Streptococcus faecalis This Klebsiella strain proved to be a very strong penicillinase producer (Table 6) and the urine became almost void of penicillin

Bacteria in sputum (Table 4) Examination of sputum for bacteria was carried out in eight patients Before injection of penicillin five of these had Pfeiffer bacteria in their sputum These bacteria had not

first penicillin injec

penicillin treatment

nonstrable in any of

the patients However in patients Nos 2 and 3 these bacteria could be found again eight days later In patient No 13 Pfeiffer bacteria were found 12 hours after the second injection even though these bacteria had not been demonstrated previously

Pneumococci were found in the sputum of four patients type 6 in three cases and type 22 in one In two patients the pneumococci had disappeared from the sputum already one hour after the first injection of penicillin in the two other patients 12 16 hours after the first injection Pneumococci type 19 were demonstrated in the sputum of patient No 2 eight days later

Gram negative rods (Coli Coli Klebsiella group) were seen during and after treatment of patients Nos 3 and 4 and Candida albicans in two patients (Nos 4 and 13) Seven of the patients housed Gram negative cocci before treatment and these persisted despite the administration of penicillin The non haemolytic streptococci found before the penicillin administration generally disappeared after the first or second injection but reappeared on cessation of penicillin treatment

TABLE 4

Bacterial Strains in Sputum before and after i m Injection of G Penicillin

Pt No	Dose of G pen in units	Strains in sputum					
		Before pen inj	1 hour after 1st inj	12 16 hour after 1st inj	1 hour after 2nd inj	12 hours after 2nd inj	After 8 days
2	5 mill × 2	Pfeiffer G coccus Str non hem	Pfeiffer G coccus Str non hem	Pfeiffer G coccus	G coccus	sterile	Pfeiffer G coccus Str non hem Pn 19
3	5 mill × 2	Pfeiffer Pn 22 G coccus	Pfeiffer G coccus Str non hem	G coccus	G coccus	G coccus	Pfeiffer G coccus Str non hem Coli Klebs
4	5 mill × 2	Staph aur G coccus	Staph aur G coccus	G coccus Coli	G coccus Coli	Coli Cand alb	—
6	5 mill × 2	Pfeiffer Pn 6 Cand alb	Pfeiffer Pn 6 Cand alb	—	Cand alb	—	—
9	10 mill × 2	Pfeiffer Pn 6 G coccus Str non hem	Pfeiffer G coccus Str non hem G rod	—	—	—	—
11	10 mill × 2	Pn 6 G coccus Str non hem Cand alb Staph aur (few)	Pn 6 (few) G coccus Cand alb	G coccus Cand alb	G coccus Cand alb	G coccus Str non hem Cand alb	
12	20 mill × 2	Pfeiffer G coccus Str non hem	Pfeiffer G coccus Str non hem	Pfeiffer G coccus Str non hem	G coccus Str non hem	—	
13	20 mill × 2	Str hem Str non hem G coccus	Str hem (few) Str non hem G coccus	sterile	sterile	Str non hem Pfeiffer Cand alb	

G = Gram negative — = Not examined

that, judged by the sensitivity test, it should be possible to eliminate all of the Gram-positive cocci with million-units-doses of penicillin The same applies to the strains of Pfeiffer and Proteus isolated Klebsiella were resistant to penicillin, even in million-units-doses, while the Coli and Gram-negative non-fermenting rods included strains which were found sensitive or resistant to large doses of penicillin The results of sensitivity testing of the bacteria mentioned generally corresponded to the results of bacteriological examination of urine and sputum during and following penicillin treatment The only deviation is seen in the Gram negative cocci Although all of the seven strains *in vitro* were sensitive to million-units doses of penicillin, these bacteria remained demonstrable in the sputum of a number of the patients after administrations of large amounts of penicillin (Table 4)

TABLE 6

Sensitivity to G Penicillin (Determined by the Tablet Method) of 46 Strains Isolated from the Urine or Sputum of 14 Patients

Bacterial strains		No of strains	Sensitivity to G penicillin							
			Normal dose Tablets of 25 units				Million unit dose Tablets of 1000 units			
			+++	++	+	0	+++	++	+	0
Gram pos cocci	Pneumococci	4	4	–	–	–	4	–	–	–
	Haem strept	1	1	–	–	–	1	–	–	–
	Non haem strept	6	5	1	–	–	6	–	–	–
	Strep faecalis	3	–	–	3	–	3		–	–
	Staph aureus	3	1	–	2	–	3	–	–	–
	Staph albus	2		–	2	–	2	–		–
Gram neg cocci	Gram neg cocci not further defined ..	7	3	–	4	–	7	–	–	–
Gram neg rods	Coli	4	–	–	1	3	3	–	–	1
	Klebsiella	2		–	–	2	–		–	2
	Proteus	1	–		1	–	1	–	–	–
	Pfeiffer	4		–	4	–	4	–		–
	Gram neg non fermenting rods	7		–	–	7	2	–	1	4
Fungi	Candida albicans	2		–	–	2		–	–	2

+++ sensitive ≥ 28 mm
+ 23 27 mm
10-22 mm
≤ 9 mm

Binding of Penicillin in Urine and Serum

As mentioned, all of the urine specimens were examined in three dilutions in order to achieve inhibition zones with one of these by the cup method Since the penicillin content in the material is reduced after storage, the test should give a readable result at the first test

On the whole, it can be stated that bacteria which by a sensitivity test are shown to be sensitive to million-unit-doses of penicillin cannot be demonstrated in sputum after treatment for 24 hours with two injections of 5, 10 or 20 million units of G-penicillin (pneumococci, haemolytic streptococci, Staphylococcus aureus, non-haemolytic streptococci, Pfeiffer) On the other hand, the more resistant bacteria (Gram-negative cocci, Candida albicans, Gram-negative rods) remain in the sputum

Ability of the Bacteria to Produce Penicillinase (Table 5)

Of the Gram-positive and Gram-negative cocci isolated from urine or sputum, it is only within the staphylococci group that penicillinase-forming bacteria are found Two strains of Candida albicans did not form penicillinase Findings varied as regards the Gram-negative rods None of the Pfeiffer or Proteus strains, and only one out of four Coli strains, formed penicillinase One of the two Klebsiella strains showed *strong penicillinase formation (urine from patient No 6)*, while the other formed penicillinase to a smaller degree All grades of penicillinase formation were seen in the non-fermenting strains which were not classified in detail

TABLE 5
Production of Penicillinase by 46 Bacterial Strains

Bacterial strains		No of strains	Penicillinase				
			+++	++	+	(+)	0
Gram pos cocci	Pneumococci	4					4
	Hem strept	1	–				1
	Non hem strept	6					6
	Strept faecalis	3					3
	Staph aureus	3	2				1
	Staph albus	2	–		2		
Gram neg cocci	Gram neg cocci not further defined	7	–				7
Gram neg rods	Coli	4	1				3
	Klebsiella	2	1		1		
	Proteus	1					1
	Pfeiffer	4					4
	Gram neg non fermenting rods	7	1		1	2	1
Fungi	Candida albicans	2					2

Sensitivity of the Strains to G-Penicillin (Table 6)

All of the strains isolated from urine and sputum were examined for sensitivity to penicillin by means of the tablet method It will be seen

data) gave one million units of G penicillin i m to six persons and found half an hour later the following concentrations in the blood 10, 25, 25, 30 30 40 units/ml

In the present work the concentrations of penicillin in serum, urine and sputum were measured at various intervals after intramuscular injections of doses of 5, 10, or 20 million units In serum the concentrations found one hour after injection were 30–130, 100–400, and 100–500 units/ml respectively At the same time the concentrations in urine were mostly about 10,000 units ml but up to 100,000 units ml were observed, while the concentrations in sputum were from 0 5–4 units ml

Bacterial strains being resistant to treatment with ordinary doses of penicillin (300–400,000 units 3–4 times daily) may be sensible to million unit doses During treatment with large doses of penicillin the bacterial flora in urine and sputum has been followed Fair correlation is found between the sensitivity testing and the results of the treatment

The sensitivity tests were carried out with a tablet method (*Lund* 1957) using tablets of 25 and 1000 units of G penicillin All strains showing + or + + with the 25 unit tablet were found to be sensitive (+ + +) with the 1000 unit tablet, whereas the strains giving no zone with the 25 unit tablet, might be sensitive or resistant with the 1000-unit tablet By the tablet method it has been found that all strains of Streptococcus faecalis, Proteus and Pfeiffer were resistant (+) to normal dose of penicillin but sensitive to million unit doses

By treatment with penicillin in million unit-doses the urine of all patients but one became sterile This patient had an infection with a Klebsiella producing penicillinase

The sputum of the patients did not—or for a short time only—become sterile After the treatment bacteria are found in the sputum, either the same strains as before or new ones During and following treatment with penicillin Gram negative rods are quite often present in the sputum Hence it seems rather doubtful, whether an antibiotic treatment of a chronic infection of the lung is of any benefit The sensitive strains are reduced or eradicated, but a reinfection or secondary infection easily takes place, most often with more resistant strains

SUMMARY

After i m injection of G penicillin in doses of 5, 10 or 20 million units to 14 patients the concentration is measured in serum, urine and sputum The effect of the treatment on the bacterial flora in urine and sputum is followed The isolated strains are examined for penicillinase production and for sensitivity to G penicillin (tablet method) It is observed that penicillin in serum and urine probably is bound reversibly

The results obtained with three dilutions were found to vary The higher the dilution the greater the penicillin values This was most pronounced in tests on urine, but was also seen when serum with a large content of penicillin was examined The more penicillin the specimen contained, the more pronounced was the phenomenon described This is illustrated by the values for penicillin content in urine and serum in patient No 5 The figures are the average of double determinations carried out on the first and second day after the specimens were received (Table 7)

TABLE 7
Concentration of Penicillin in Urine and Serum after i m Injection 5 Million Units of G penicillin (Patient No 5)

Specimen taken	Urine			Serum		
	1 100	1 1000	1 10 000	1 1	1 10	1 100
1 hour after 1th inj	5 000	7 000	30 000	25	30	50
15 hours after 1th inj	500	1,000	4 000		< 0 03	
1 hour after 2nd inj	5 000	9 000	40 000	30	40	70

Figures indicate U/ml of G penicillin
The values are the average of double determinations

The most reliable results are supposed to be found with the dilution giving an inhibition zone of middle-size Therefore it has been chosen to give the answer as the result of the dilution in the middle of the three used in the experiment

This dilution phenomenon is due possibly to a reversible fixation of penicillin to some substances in urine and serum Fixation of penicillin to serum albumin has been described by *Chow & McKee* (1945), *Beyer* (1950) and *Marner & Lund* (1957) Only one of the patients (No 8) had albumin in the urine, but the phenomenon was found by measuring all urines containing penicillin, and hence cannot be due to fixation to that substance No previous reports have been found concerning the fixation of penicillin in urine, and this aspect has not been studied further in the present work

DISCUSSION

Few authors only have studied the concentration of penicillin in serum and urine after administration of more than one million units *Tucker & Eagle* (1948) found a concentration of 35 units/ml half an hour after intramuscular injection of 1 2 million units of G-penicillin *Boger* (1950) found about 20 units/ml one hour after 1 million units and *Knothe et al* (1956) found an average of 4 6 units/ml after one million units given 11 times a day, and 20 30 units/ml after ten doses of 1 8 million units in one day *Marner & Lund* (1957, unpublished

FROM THE DEPARTMENT OF BACTERIOLOGY KAROLINSKA INSTITUTET STOCKHOLM 60 SWEDEN

LABORATORY EQUIPMENT FOR GROWING AEROBIC BACTERIA IN HIGH YIELDS

By

L EDEBO C G HEDÉN, T HOLME and B ZACHARIAS

Received 22.xi 61

Batch cultivation of aerobic bacteria on a laboratory scale usually presents no great problems However, if high yields are desired, special attention must be paid to the aeration efficiency and to the pH-control A study of the influence of the aeration efficiency on the yield of *Serratia marcescens* has been published (*Smith & Johnson* 1954), but many bacteria seem to be far more sensitive to changes in pH than this organism In the present paper a detailed description will be given of a one-litre culture vessel with automatic pH control and highly efficient aeration The equipment was developed with the aim of providing worker in biochemistry and cell physiology with a simple and reliable [illegible]

Apparatus Design

The equipment was designed for a liquid volume of one litre The culture vessel was made of a Pyrex glass tube with an inner diameter of 70 mm (Fig 1) The neck was closed by a perforated rubber stopper, with glass tubes for air inlet (B) and outlet (C), for addition of alkali or acid (A) and for sample taking (not indicated in the figure) Samples could also be taken with the aid of a special attachment described earlier (*Heden & Holme* 1960) Two glass tubes were fused into the side of the vessel one of them (D) being open to provide a fitting for a pH electrode and the other (E) constituting a pocket for a regulating thermometer

Aeration

Air [illegible]
cylind [illegible]

Our t[illegible] to Miss *Berit Lindholm* for her skilled technical assistance
This work was aided by a grant from the *Swedish State Medical Research Council*

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Apparatus Design

The equipment was designed for a liquid volume of one litre The culture vessel was made of a Pyrex glass tube with an inner diameter of 70 mm (Fig 1) The neck was closed by a perforated rubber stopper, with glass tubes for air inlet (B) and outlet (C), for addition of alkali or acid (A) and for sample taking (not indicated in the figure) Samples could also be taken with the aid of a special attachment described earlier (*Heden & Holme* 1960) Two glass tubes were fused into the side of the vessel one of them (D) being open to provide a fitting for a pH electrode and the other (F) constituting a pocket for a regulating thermometer

Aeration

Air was introduced into the culture through two sintered Pyrex glass cylinders (F) 10×20 mm with a pore size of 40-60 μ (Corning Glass

Our thanks are due to Miss Berit Lindholm for her skilled technical assistance
This work was aided by a grant from the Swedish State Medical Research Council

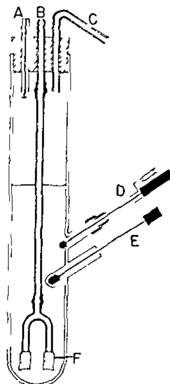


Fig 1
Culture vessel A – glass tube for acid or alkali addition B – glass tube for inlet air C – air outlet D – pH electrode E – regulating thermometer F – gas distribution cylinders

Works, New York) For practical reasons the glass tube for the inlet air was divided into three sections, connected with short pieces of latex rubber tubing The air flow was measured by a gas flowmeter Air filtration was effected by glass wool (PF-105 Owens-Corning Fiberglass Corp, *Toledo, Ohio*)

TABLE 1
Aeration Efficiency as Estimated by the Sulphite Oxidation Method

Cylinders tested	Air flow (litres per min)	Effective aeration (mM O_2 per litre an I hour)
A	1	190
B	1	192
C	1	194
B+C	1	218
A+B+C	1	227
A	2	251
B	2	271
C	2	284
B+C	2	480
A+B+C	2	490

Liquid volume 1 litre Three gas distribution cylinders were tested marked A B and C

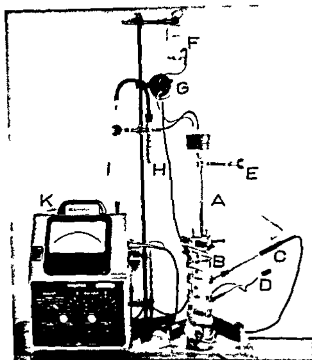


Fig 2

High yield cultivation unit A culture vessel B heating tape C – pH electrode D regulating thermometer F KCl supply for electrode F acid or alkali supply G magnetic valve H air filter I air flow meter K titrator

Aeration Efficiency

With the shape of the culture vessel and type of gas dispersion employed no mechanical agitation was necessary to obtain good aeration In Table 1 data are given for the aeration efficiency as estimated by the sulphite oxidation method (*Cooper, Fernstrom & Miller* 1944)

Control of pH

An automatic titration unit was used (type TTT 1 Titrator plus type MNV 1 magnetic valve manufactured by Radiometer Copenhagen) A combined glass calomel electrode (type GK 2021 C) was fitted into the vessel through the open side tube and sealed to it by means of a short piece of latex rubber tubing (D) Shifting from acid to alkali titration had to be performed manually

Temperature Control

A standard regulating thermometer was fitted into the glass pocket fused into the wall of the culture vessel (F) A few drops of glycerol

were put into the pocket before inserting the thermometer to improve heat transfer A 36-watt heating tape of 60 cm length (Electrothermal Engineering Ltd) was wound around the vessel and connected to an electronic relay (Sunvic type EA 4)

Operation

The empty culture vessel and the rubber stopper with the aeration tube were sterilized separately The electrode was sterilized in 70 per cent ethanol containing 0 05 per cent sulphuric acid or in 10 per cent formaldehyde After sterilization, the pH-electrode was aseptically immersed in the media through the side tube of the culture vessel, and fixed with a wire around the latex rubber seal to insure against leakage Inoculated medium was then poured into the vessel and the rubber stopper fitted into its neck Air was then introduced, the heating tape connected and the pH checked A latex rubber tube for acid or alkali was fitted into the magnetic valve and attached to the vessel, and the automatic pH-control was finally put into operation

Usually a silicone antifoam was added to the growth medium before sterilization (*Gergle et al* 1957) This proved to be sufficient in most instances If antifoam addition was required during growth, this was done from a 10-ml syringe filled with sterilized antifoam, and connected to a Luer-lok needle perforating the rubber stopper in the neck of the culture vessel Mechanical foambreaking could also be provided as described below

Mechanical Agitation

A modification of the culture vessel, using mechanical stirring and foambreaking, was also tested (Fig 3) In this case, the head of the culture vessel was made of glass and fitted with a ground flange (C) Two gas distribution cylinders (H) were melted into the bottom of the vessel The shaft consisted of an axial glass tube and was provided with a transverse foambreaker rod (D) Two bearings of teflon, one at the bottom, the other at the top, supported the shaft Around this, below the top bearing, a ceramic compound magnet (B) was firmly fitted[1] At this level the top portion of the cultivation tube tapered down to a dimension which just permitted the cylindrical magnet to rotate freely The magnet is four-polar and thus surrounded by a magnetic field not unlike a simple cog-wheel As a consequence it can be made to rotate with the aid of an identical magnet (A) spinning outside the glass tube High torque and speed (1200 r p m and more) can be achieved with this system

---

1 This type of corrosion and sterilization resistant magnet (Ferroxdur 100 Vk 37504 Philips Comp Eindhoven the Netherlands) which is used in certain makes of bicycle dynamos is quite cheap

Mechanical stirring diminished the fluctuations in pH and the foam-breaker decreased considerably the need for chemical antifoam Despite the fact that stirring gives longer travelling distance to bubbles, and created turbulent shear, no great increase in the oxygen absorption rate was observed in the present system

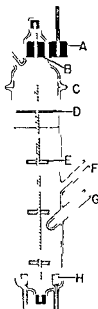


Fig 3

Culture vessel equipped with mechanical agitation A – outside Ferroxdur magnet B inside magnet C ground flange D – foambreaker F – impellers F – side tube for pH electrode G pocket for regulating thermometer H – air distribution cylinders

EXPERIMENTAL

The cultivation experiments presented below were made with a vessel without mechanical stirring Silicone antifoam was added to the media before sterilization The culture volume was one litre in all experiments Growth was followed by nephelometric measurements and the cultiva tion was discontinued when no turbidity increase could be recorded during an observation period of two hours Two per cent ...

Dry weight determinations were made on washed cells after drying to constant weight at 105° C The dry weight of the high molecular weight compounds released during growth was determined on the cell free supernatant after dialysis overnight in the cold

Strains

The strains used were *Escherichia coli B, Salmonella typhimurium*, a virulent strain isolated during an epidemic in 1952, *Bacillus megaterium*, strain M, and *Azotobacter vinelandii*, strain 0

Media

The following four media were used (concentrations in g per litre)

1	Glucose	40	2	Lactic acid	20
	NH_4 succinate	1		Succinic acid	2.6
	NH_4Cl	5		NH_4OH	4
	$Na_2HPO_4 \cdot 2H_2O$	7.5		KH_2PO_4	7
	KH_2PO_4	3		K_2HPO_4	3
	$Na_2SO_4 \cdot 10H_2O$	2.5		$Na_2SO_4 \cdot 10H_2O$	2.5
	$MgSO_4 \cdot 7H_2O$	0.2		$MgSO_4 \cdot 7H_2O$	0.2
3	Glucose	10	4	Glucose	30
	NH_4Cl	1		KH_2PO_4	0.1
	$Na_2HPO_4 \cdot 2H_2O$	7.5		$MgSO_4 \cdot 7H_2O$	0.4
	KH_2PO_4	3		Na citrate	0.2
	$Na_2SO_4 \cdot 10H_2O$	2.5		Ca acetate	0.2
	$MgSO_4 \cdot 7H_2O$	0.2		NaCl	0.1
	Difco yeast extr	2		$FeSO_4 \cdot 7H_2O$	0.01
	Difco cas ac	20		$Na_2MoO_4 \cdot 2H_2O$	0.001

Escherichia coli and *Salmonella typhimurium* were grown both on medium 1 and medium 2 with similar results *Bacillus megaterium* was grown on medium 3 *Azotobacter vinelandii* was grown on a nitrogen-free medium used by *Nicholas et al* (1960), with the glucose concentration increased to 30 g per litre (medium 4)

Glucose and magnesium sulphate were sterilized separately One ml of the following trace elements solution was added to media 1–3 before inoculation $CaCl_2$ 0.5 g per litre, $FeCl_3 \cdot 6H_2O$ 16.7, $ZnSO_4 \cdot 7H_2O$ 0.18, $CuSO_4 \cdot 5H_2O$ 0.16, $MnSO_4 \cdot 4H_2O$ 0.15, $CoCl_2 \cdot 6H_2O$ 0.18, and EDTA 20.1 g per litre Succinate was added to media 1 and 2 to decrease the length of the lag phase This could also be accomplished by mixing the air with 5 per cent carbon dioxide during the first two hours of the cultivation period The pH of the media was adjusted to 7.4 before sterilization

Cultivation Conditions and Results

Precultures of *E coli* B and *Salmonella typhimurium* were grown for 14 hours in Erlenmeyer flasks on a rotary shaker Cells were inoculated with the growth from two nutrient agar slants *Azotobacter vinelandii* was precultivated overnight in 50 ml of the same medium as was used for the final cultures No pH control was needed in the *Azotobacter cultures*

When cultivating *Salmonella typhimurium*, air was drawn through the cuture by suction for safety reasons Before entering the water-jet, the outgoing air from the culture was bubbled through concentrated

sulphuric acid and then led through a copper pipe heated with a heating tape

Cultivation conditions and yields obtained in different experiments are given in Table 2

TABLE 2

Cultivation Conditions and the Yields Obtained

		Cultivation time (hours)	Temperature (°C)	Air flow (l/min)	pH	Yield dry weight in grams per litre	
						cells	supernate
E coli B	1	18	37	2	7.1	10.0	2.2
	2	18	37	2	7.1	12.1	2.0
Salmonella typhimurium	1	18	37	2	7.1	15.5	1.1
	2	18	37	2	7.1	16.5	1.7
Bacillus megaterium		12	37	1	7.1	5.0	–
Azotobacter vinelandii	1	18	30	1	no contr	13.3	–
	2	18	30	1	no contr	15.9	–

DISCUSSION

Usually it is assumed that mechanical agitation is required to obtain an efficient aeration in a bacterial culture In the present study, it was shown that a good gas dispersion combined with a high liquid head could provide an effective aeration superior to most stirred laboratory fermentors This made it possible for us to build a cultivation apparatus, which had the simplicity of ordinary laboratory equipment, but still permitted the production of aerobic bacteria in high yields However, since stirring may be desired, for instance to control the morphology of a mycelium (*Dion et al* 1954), we also described a simple magnetic transmission, well suited for bacterial cultures This transmission also permits the introduction of a mechanical foam-breaker, as this may be necessary in some cases At high cell densities, considerable amounts of foam were formed even if the culture was grown on a purely synthetic medium In these cases chemical antifoam had to be added in substantial amounts This is known to decrease oxygen transfer (*Deindoerfer & Gaden* 1955, *Solomons & Perkins* 1958) It was repeatedly found that the addition of antifoam during the later stages of growth decreased the yield Better results were obtained when antifoam was added to the media before sterilization Cultures of *Bacillus megaterium* had to be interrupted at an earlier stage than others because of intense formation of foam, in spite of the chemical antifoam added Addition of antifoam during later stages of cultivation immediately stopped the growth of this organism

With the media used, either an acid or an alkaline reaction was produced throughout the culture cycle Therefore, we consider a manual control of the shift from upwards to downwards titration sufficient

When growing pathogenic bacteria a reduced air pressure was maintained over the culture for safety reasons This did not decrease the aeration efficiency, as estimated by the sulphite oxidation method The basic equipment described above has, after appropriate modifications, also been used for the continuous culture of *Salmonella typhimurium* (*Holme & Edebo* 1961)

SUMMARY

A unit designed for small scale cultivation of bacteria is described Efficient aeration and automatic pH control makes it possible to obtain high yields of aerobic bacteria The unit is inexpensive, and its simplicity makes it ideal for workers with but little knowledge of cultivation techniques It is easily modified for mechanical foambreaking and stirring, and for continuous culture

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BEHAVIOUR OF E.COLI ANTIGENS IN SEXUAL RECOMBINATION

By

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The *Enterobacteriaceae* have surface antigens and flagellar antigens which can be analysed by simple methods The stability of these antigens seems to be great and no other group of bacteria has so convincingly been subdivided serologically as the Gram negative intestinal bacteria

A genetic analysis of the fate of these antigens in sexual recombination experiments is therefore very tempting Unfortunately, the strains used hitherto in such experiments, *e g* Coli K12, Coli B and Coli C, are all auto agglutinable and thus not very useful in immunogenetic investigations

Furness & Rowley (1955) and *Furness* (1958) reported crossing experiments between a motile K12 F^{+} strain and a non flagellated *E coli* strain B F In crosses using either motility or tyrosine as the selective marker of the *F* parent, they found that the motile recombinants always had the same H antigen as the male parent *Calef & Cavalli* (1954) mention mucoid recombinants from a cross *E coli* B × K 12 which might be determined by a pair of complementary genes serological examinations were not carried out *Schlegel Oprecht & Fey* (1959) and *Schlegel Oprecht* (1959) examined the capsule antigen in a cross between a K12 *Hfr* strain and an *E coli* strain with K antigen of the A type The authors suggested that two different genes were responsible for the development of a capsule

In a previous paper *Ørskov & Ørskov* (1961a) reported on the fertility of *E coli* antigenic test strains in crossing experiments with K12

The present study describes sexual recombination experiments involving such fertile *E coli* antigen test strains

These studies were initiated at the *Department of Genetics* University of Wisconsin Madison Wis The work was supported by research grants (to Professor Lederberg) from *National Science Foundation* and from the *National Cancer Institute* (C 2157) *U S Public Health Service* I Ørskov [illegible]

MATERIAL AND METHODS

Crossing technique Equal amounts of fresh broth were added to overnight broth cultures The cultures were incubated for 3 hours at 37° C in roller For $F^+ \times F^-$ crosses cultures were mixed directly on minimal medium (FMS), in *Hfr* $\times$ F^- crosses the young broth cultures were mixed and diluted appropriately immediately before plating Recombinants were scored after 48 hours' incubation at 37° C Recombinants were isolated on complete medium (FMB) and single colonies were restreaked on media suitable for determining the parental character of the different markers

TABLE 1
Strains Used

Source	Designation	Markers	Serotype
K12	W 6	F met lac mal str^{s}fla$^+$H48 O K$^-$	*O K? H48
W G4	W 1611	F leu try lac mal sor$^-$str^{r}fla H16 O25$^+$K	§O25 K (L) nm
W G4	W 3697	F leu try lac mal sor$^-$str^{r}fla H16 O25 K	O25 K (L) nm
W G4	W 3703	Hfr leu try lac mal sor$^-$str^{r}fla H16 O25 K	O25 K (L) nm
H509a	W 3462	F his lac$^+$mal rha$^+$str^{s}fla$^+$H2 O100+K+	†O100 K$^+$(B) H2
H509a	W 3473	F his ser lac$^+$mal$^+$rha$^+$str^{s}fla$^+$H2 O100+K+	O100 K$^+$(B) H2
H509a	*W 3480*	*F$^+$ his isl lac$^+$mal rha$^+$str^{s}fla$^+$H2 O100 K+*	*O100 K$^+$(B) H2*
H511	W 3451	F pro thr rha fla H8+O102+K$^+$	O102 K$^+$(B) H8
H511	W 3442	F pro met rha fla$^+$H8 O102+K$^+$	O102 K$^+$(B) H8
Bi449/42	D140	F his pro lac$^+$mal sor str^{s}fla$^+$H? O9+K26	O9 K26(A) nm
Bi449/42	D162	F his pro lac$^+$mal sor$^-$str^{s}fla$^+$H? O9+K26	O9 K (A) nm
A12b	D148	F his met lac$^+$mal str^{s}fla$^+$H10 O6+K54	O6 K54(L) H10
A12b	D149	F his ser$^-$lac mal str^{s}fla H10 O6 K54+	O6 K54(L) H10
A12b	D154	F his met lac mal str^{s}fla$^+$H10 O6 K54	O6 K (L) H10
C24/55	W 3706	F his lac mal str^{s}fla H32 O26+K60	O26 K60(B) H32

* All K12 strains are characterized as rough no O antigen has been detected up till now The presence of an ordinary K antigen is doubtful The H antigen is numbered H48 (*Ørskov & Ørskov* 1960 b)
§ Indicates O antigen 25 no K antigen and non motile The K antigen originally present in W G4 was of the L type
† Indicates O antigen 100 the presence of a K antigen of the B type not yet numbered H antigen 2

met = methionine leu = leucine try = tryptophane his = histidine ser = glycerine serine isl = isoleucine pro = proline thr = threonine lac = lactose mal = maltose sor = sorbitol rha = rhamnose str^s or str^r = streptomycin sensitive or resistant

Strains used Strains with the prefix W were developed in Dr *Lederberg*'s laboratory Department of Genetics University of Wisconsin Madison Wisconsin and those with prefix D at The International Escherichia Centre Statens Seruminstitut Copenhagen Denmark In several of the crossing experiments reported here one of the parents is an auxotrophic mutant from the W G4 line These strains contain O antigen 25 and K antigen of the L type here named K(W 1611) or no detectable K antigen K form Using conventional methods strains from the W G4 line are immotile even after many subcultures in semifluid agar no motile organisms could be isolated Flagella staining *a m Leifson* did not show any flagella For further data regarding *W 1611 and W 3703 see Ørskov & Ørskov* (1960 a) W 3480 W 3473 and W 3462 are auxotrophic mutans of the test strain for *E coli* O antigen 100 The markers *histidine isoleucine* and *glycerine serine* have been introduced with the help of standard methods (*Lederberg* 1950) Similarly D140 is an auxotrophic mutant derived from the test strain for *E coli* K antigen 26 (A antigen) D162 is a spontaneous K mutant from D140 *i e* without detectable capsule isolated from a broth agar plate as a non mucoid sector in a mucoid colony D148 is a *histidine methionine* mutant derived from the antigenic test strain of *E coli* K54 (L antigen) and

D154 a spontaneous mutant of that strain For further information regarding serological technique and the antigenic constitution of coli bacteria the reader is referred to *Ørskov & Ørskov* (1960) and *Kauffmann* (1954)

Media For crosses the minimal medium EMS (*Lederberg* 1950) was employed For scoring of fermentative markers and sensitivity to streptomycin EMB plates with the appropriate supplementations were used For serological analysis ox heart broth infusion was used for plates (1.6 per cent agar) and beef broth for fluid medium In both cases 1 per cent peptone 0.3 per cent NaCl and 0.2 per cent $Na_2HPO_4 \cdot 12H_2O$ were added

For motility determination U tubes with semifluid agar (0.225 per cent agar) were used The H determinations were carried out as immobilization tests (*Ørskov* 1954)

TABLE 2

Transfer of Unselected Markers in the Cross

W6F thr leu met lac mal strsfla H48 O

×

W1611F thr⁻leu⁻met lac mal strrfla H16 O25

Unselected markers transferred from F parent

lac⁺	mal	fla	O	No	Per cent
—	—	—	—	19	35.2
+	—	—	—	13	24.1
+	+	—	—	2	3.5
+	+	+	—	1	1.9
—	—	+	—	7	13.0
+	—	+	—	6	11.1
—	+	—	—	4	7.4
—	—	—	+	1	1.9
+	—	+	+	1	1.9
Total number of recombinants				54	

+ indicates transfer of unselected markers from F parent
Recombinants selected on non supplemented medium

EXPERIMENTAL RESULTS

As already mentioned *Furness & Rowley* (1955) found only the paternal flagellar antigen among the progeny in a cross K12 *F* × coli B *F* In the cross W6 *F* × W1611 *F* (Table 2) we found that 52 out of 54 recombinants had the same O antigen as the *F* parent It will be seen that 15 or 27 per cent of recombinants were motile It was further found that these motile recombinants all had flagellar antigen H16 and not H48 which is the H antigen of K12 strains As mentioned ...

... motility factor comparable to the *fla* factor found in the *Salmonella* group in phage mediated transduction (*Zinder & Lederberg* 1952 *Stocker Zinder & Lederberg* 1953) It is apparent from the results that the *fla* locus and the locus for H specificity are not transferred together In the cross W3480 *F* × W1611 *F* a similar transfer of a *fla* locus was observed In other crosses where a strain from from the WG4 line was part as *F* parent segregation for

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TABLE 1

Strains Used

Source	Designation		Markers	Serotype
K12	W6	F	met lac mal str^{s}fla H48+O K	*O K? H48
WG4	W1611	F	leu try lac mal sor str^{r}fla H16 O25+K	§O25 K (L) nm
WG4	W3697	F+	leu try lac mal sor-str^{r}fla H16 O25 K	O25 K (L) nm
WG4	W3703	Hfr	leu try lac mal sor str^{r}fla H16 O25 K	O25 K (L) nm
H509a	W3462	F	his lac mal rha+str^{s}fla+H2 O100+K+	†O100 K+(B) H2
H509a	W3473	F	his ser lac mal rha+str^{s}fla+H2 O100+K+	O100 K+(B) H2
H509a	W3480	F	his isl lac mal rha+str^{s}fla+H2+O100+K+	O100 K+(B) H2
H511	W3451	F	pro thr rha fla+H8+O102+K+	O102 K+(B) H8
H511	W3442	F	pro met rha fla H8 O102+K+	O102 K+(B) H8
Bi449/42	D140	F	his pro lac+mal sor str^{s}fla H? O9+K26	O9 K26(A) nm
Bi449/42	D162	F	his-pro lac+mal sor-str^{s}fla H? O9+K26	O9 K (A) nm
A12b	D148	F	his met lac+mal str^{s}fla+H10 O6+K54	O6 K54(L) H10
A12b	D149	F	his ser-lac+mal str^{s}fla+H10 O6 K54+	O6 K54(L) H10
A12b	D154	F	his met lac+mal str^{s}fla+H10 O6 K54	O6 K (L) H10
C24/55	W3706	F	his lac mal str^{s}fla H32 O26+K60	O26 K60(B) H32

* *All K12* strains are characterized as rough no O antigen has been detected up till now The presence of an ordinary K antigen is doubtful The H antigen is numbered H48 (*Ørskov & Ørskov* 1960 b)

§ Indicates O antigen 25 no K antigen and non motile The K antigen originally present in WG4 was of the L type

† Indicates O antigen 100 the presence of a K antigen of the B type not yet numbered *H* antigen 2

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TABLE 3

Transfer of Unselected Markers in the Cross

W3703Hfr his$^+$ ser$^+$ leu try lac mal str^r fla H16 O25$^+$ k$^-$
×
W3473F his ser$^-$ leu try$^+$ lac$^+$ mal$^+$ str^s fla$^+$ H2 O100$^+$ k$^+$

45 recombinants examined
Unselected markers transferred from F+ parent

	his$^+$	lac	str^r	fla	H16$^+$	O25$^+$	k	No	Per cent
	—	—	—	+		—	—	2	4 4
	—	—	—	—	—	—	—	23	51 1
	—	—	+	—	—	—	—	1	2 2
	—	—	—	—	+	—	—	1	2 2
	—	+	—	—	—	—	—	1	2.2
	+	—	—	—	+	+	+	11	24 4
	+	—	—	—	—	+	+	5	11 1
	+	—	—	+		+	+	1	2 2
No	17	1	1	3	12	17	17		
Per cent	37 8	2 2	2 2	6 7	26 4	37 8	37 8		

+ indicates transfer of unselected markers from F+ parent
Recombinants selected on minimal medium supplemented with histidine and tryptophane

TABLE 4

Transfer of Unselected Markers in the Cross

W3703Hfr his$^+$ pro$^+$ leu try lac$^-$ mal sor$^+$ str^r fla H16 O25$^+$ k
×
D140F his pro leu$^+$ try$^+$ lac$^+$ mal$^+$ sor str^s fla H? O9$^+$ k26

53 recombinants examined
Unselected markers transferred from F+ parent

	his$^+$	lac	mal	sor$^+$	O25$^+$	k$^-$	No	Per cent
	—	—	—	—	—	—	8	15 1
	—	+	—	—	—	—	28	52 8
	—	—	—	+	—	—	1	1 9
	—	+	+	—	—	—	1	1 9
	—	+	—	+	—	—	5	9 4
	+	+	—	+	+	+	5	9 4
	+	+	—	—	+	+	5	9 4
No	10	52	1	11	10	10		
Per cent	18 9	98 1	1 9	20 8	18 9	18 9		

+ indicates transfer of unselected markers from F+ parent
Recombinants selected on minimal medium supplemented with histidine

sponding part of the male chromosome, adjacent to the *his* marker, containing the O antigen determinant for O 25 but no determinant for a socalled B antigen More than 90 per cent of recombinants were *fla*$^+$ and of these 29 per cent belonged to H16 and the rest to H2

A long series of other crosses between W3703 and other F strains not reported here have consistently confirmed that the genetic determinants for the histidine synthesis and for the O antigen, and to a lesser degree for the H antigen specificity, are closely connected

the *fla* factor was similarly observed (see below) In two further crosses which shall not be described in detail, W3480 F^+ × W3451 *F* and W3480 F^+ × W3442 F^-, no transfer of antigenic markers from F^+ to *F* was detected

Only when histidineless mutants were introduced as females transfer of other antigenic markers was found

In the crosses: W3697 F^+ × W3473 *F* and W3697 F^+ × W3706 *F* serological examination of the progeny gave uniform results Both crosses were carried out on unsupplemented minimal medium In the first, all recombinants (33) had O antigen 25 and 22 of 31 motile recombinants belonged to H16 In the other cross, 54 recombinants were examined, 53 of which carried the O antigen of the male parent 54 recombinants were motile and 13 belonged to H16, *i e* they had received H specificity from the F^+ parent

The outcome of the above-mentioned crosses suggested strongly that the genetic determinant for the O antigen specificity was transferred along with the histidine locus An *Hfr* mutant (*Hfr* for histidine) was therefore sought among fertile *E coli* strains which could be converted to the F^+ state After some unsuccessful attempts with the type strain for O group 100, efforts were made to isolate an *Hfr* culture from W3697 F^+*leu try* after uv treatment and using the replica plating technique (*Lederberg* & *Lederberg* 1952) with selection for transfer of the histidine marker This new *Hfr* strain was called W3703 It was further found that crosses between W3703 and different histidineless mutants of the antigenic type strains gave very high numbers of prototrophic recombinants corresponding to a 1000 to 10,000-fold increase in the number of recombinants

An investigation into the kinetics of this *Hfr* recombination experiment has not yet been carried out successfully, but a review shall be given here of a number of crossing experiments using this new *Hfr* system

In the cross W3703 *Hfr* (*leu try lac mal str*^{r} *fla H16*$^+$*O25*$^+$*K*) × W3462 *F* (*his lac*$^+$ *mal*$^+$ *str*^{s} *fla*$^+$ *H2*$^+$*O100*$^+$*K*$^+$), 48 out of 48 recombinants had the following constitution *leu*$^+$ *try*$^+$ *his*$^+$ *lac*$^+$ *mal*$^+$ *str*^{s} *fla*$^+$ *O25* 41 of these motile recombinants were sufficiently motile for an H determination, of these 6 belonged to H2 and the rest to H16 K antigen determinations of the recombinants showed that none of them had the K antigen of the *F* parent but closer examination of the K antigen was not carried out

The results of another typical cross involving W3703 *Hfr* can be found in Table 3 It will be seen that *his*$^+$ which in this crossing experiment, in contrast to the crosses described previously, is an unselected marker, is found in 17 recombinants and that all these have received O antigen 25 at the same time The same recombinants were found also in the K state, probably indicating that the determinants for both the O and the B antigen of the recipient strain is replaced by the corre-

It is confirmed again in the cross recorded in Table 6 that the O antigen will follow the marker for histidine to a great extent It is further apparent that 54 out of 56 recombinants receiving O25 from the *Hfr* strain have their K antigen from the *F* parent, *i e* K54(L) This is in contrast to the above described crosses between W3703 and an *F* strain equipped either with a B or an A antigen, in which cases the O25 recombinants were all K Two recombinants (Table 6) with 025 from the male parent are furnished with the K antigen of the L type present in K^+ forms of the WG4 line here named K(W1611) Reversion or reappearance of a K antigen has never been observed in a coli strain in which such antigen has been lost and especially not in strain W3703 The appearance of the type O25 K(W1611) in this cross therefore strongly suggests the existence of a genetic factor in addition to the one determining the serological specificity of the L antigen, necessary for the phenotypic expression of the L antigen The situation is therefore reminiscent of that of the coli H antigens, and we would propose calling this new genetic factor *kap* in analogy to the *fla* marker of the H antigens Thus W3703 would be *kap*$^-$K(*W1611*)$^+$ while D148 *is kap* K54$^+$ In the cross recorded in Table 7 the same strains are used except that a K mutant of D148 D154 is used as female parent Other markers

TABLE 6

Transfer of Unselected Markers in the Cross

W3703Hfr his met leu try$^-$lac$^-$mal str^{r}fla H16 O25 K
×
D148F his met leu try$^+$lac$^+$mal str^{s}fla H10$^+$O6 K54

60 recombinants examined

	his	lac	mal	str	fla	H16	O25	K	No	Per cent
	+	−	−	−	−	+	+	−	22	36 7
	+	−	−	−	−	−	+	−	7	11 7
	+	−	+	−	−	+	+	−	8	13.3
	+	−	+	−	−	−	+	−	1	1 7
	+	−	−	+	−	+	+	−	6	10 0
	+	−	−	+	−	−	+	−	2	3 3
	+	−	+	+	−	+	+	−	3	5 0
	+	−	+	+	−	−	+	−	1	1 7
	+	−	+	−	+		+	−	1	1 7
	+	+	+	−	−	−	+	−	1	1 7
	+	−	−	−	+		+	−	1	1 7
	+	−	−	−	−	+	+	K(W1611)	1	1 7
	+	−	−	+	−	+	+	K(W1611)	1	1 7
	+	−	−	−	−	−	−	−	1	1 7
	+		+	+	−	+	−	−	1	1 7
	+	−	+	+	−	−	−	−	1	1 7
	+	+	+	−	−	−	−	−	1	1 7
		−	−	−	−	−	+	−	1	1 7
No	59	2	18	15	2	42	56	0		
Per cent	98 3	3 3	30 0	35 0	3.3	70 0	93.3	0		

+ indicates transfer of unselected markers from F parent
Recombinants selected on minimal medium supplemented with histidine

In order to investigate the problem more closely, the following mating experiments between K^+ and K^- forms of the same strain as the female and W3703 as the male partner were set up —

W3703 *Hfr* × D140 *F* and W3703 *Hfr* × D162 *F*

the only difference being that D162 is a spontaneous K^- mutant isolated from D140 (Tables 4 and 5) K26 is an A antigen and it can be seen (Table 4) that no segregation of the genetic determinants for the K and O antigens are found in this cross It should be stressed also that K(W1611), *the latent K antigen of WG4 and its derivatives, has not* appeared in the progeny, nor was K26 found among the recombinants in the cross between the two K forms

TABLE 5

Transfer of Unselected Markers in the Cross

W3703Hfrhis$^+$pro$^+$leu try lac mal sor$^+$strrfla H16$^+$O25$^+$K
×
D162F his pro leu$^+$try$^+$lac$^+$mal$^+$sor str^{s}fla$^+$H? O9$^+$K26

53 recombinants examined

	his$^+$	lac	mal	sor$^+$	O25	No	Per cent
	—	—	—	—	—	11	20 8
	—	+	—	—	—	21	39 6
	—	—	—	+	—	2	3 8
	—	+	+	—	—	4	7 5
	—	+	—	+	—	6	11 3
	+	+	—	—	+	5	9 4
	+	—	—	+	+	3	5 7
	+	—	—	—	+	1	1 9
No	9	36	4	11	9		
Per cent	17 0	67 8	7 5	20 8	17 0		

+ indicates transfer of unselected markers from F^+ parent
Recombinants selected on minimal medium supplemented with histidine
Five recombinants all prototrophic (his+) and belonging to O25 were weakly motile only in one case was it possible to carry out an H determination that recombinant belonged to H16

W3703, *D140 and* D162 *are all immotile, but among* the recombinants in these two crosses a number of weakly motile recombinants was found, all among those having received the *his*$^+$ marker from the donor strain Among these prototrophic recombinants six out of ten from the cross W3703 × D140 and five out of nine from the cross W3703 × D162 were motile, but except for one from the cross W3703 × D162 belonging to H16, they were not sufficiently motile for H determination

In order to see whether the O and K antigen markers would recombine if the female parent were equipped with a K antigen of the L type, the following mating experiments were carried out —

W3703 *Hfr* × D148 *F* and W3703 × D154 *F* (Tables 6 and 7), the difference between D148 *and* D154 being that D148 has K *antigen* 54(L) and that D154 is a K form isolated as a spontaneous mutant from it

As apparent from the experiments described above in which the O antigen specificity follows the *histidine* marker, a hypothetical latent K12 O antigen might be found among recombinants from a cross between K12 F^+ and a histidineless F^- strain equipped with an O antigen.

The actual cross was carried out between W6 F^+ and W3478 F^-. 48 recombinants examined with a regard to the O antigen were all spontaneously agglutinable (rough). Even though the recombinants were scored on unsupplemented minimal medium, this result would suggest that loci for O and R antigens are situated close to each other on the coli chromosome.

In Table 8 a list is given of the different serotypes found in the crosses.

TABLE 8

List of Seroformulas of Recombinants Found in Crosses Recorded in Tables 2 to 7

	Hfr or F^+			F^-			Recombinants		
	O	K	H	O	K	H	O	K	H
Table 2	—	—	H48	25	—	—	—	—	—
							—	—	*16*
							25	—	—
							25	—	*16*
Table 3	25	—	—	100	(B)	2	25	—	—
							25	—	*16*
							25	—	2
							100	(B)	2
							100	*(B)*	*16*
							100	*(B)*	—
Table 4	25	—	—	9	26(A)	—	25	—	—
							9	26(A)	—
Table 5	25	—	—	9	—	—	25	—	—
							25	—	*16*
							9	—	—
Table 6	25		—	6	54(L)	10	25	*54(L)*	*16*
							25	*54(L)*	*10*
							25	*54(L)*	—
							25	*(B 1611)(L)*	*16*
							6	54(L)	10
							6	*54(L)*	*16*
Table 7	25	—	—	6	—	10	*25*	—	*16*
							25	—	10
							25	*(B 1611)(L)*	*16*
							25	*(B 1611)(L)*	*10*
							6	—	10

New hybrid serotypes are written in italics.

TABLE 7

Transfer of Unselected Markers in Cross

W3703Hfrhis$^+$met$^+$leu try lac mal strrfla H16 O25$^+$K

×

D154F his met leu$^+$try$^+$lac$^+$mal$^+$strsfla$^+$H10$^+$O6$^+$K54

48 recombinants examined

	his$^+$	lac	mal	strr	fla	H16$^+$	O25$^+$	No	Per cent
	+	—	—	—	—	+	+	20	41 7
	+	—	—	—	—	—	+	6	12 5
	+	—	+	—	—	+	+	7	14 6
	+	—	+	—	—	—	+	1	2 1
	+	—	+	+	—	+	+	4	8 3
	+	—	—	+	—	+	+	2	4 2
	+	+	+	—	—	+	+	1	2 1
	+	+	+	—	—	—	+	1	2 1
	* +	—	—	+	—	+	+	2	4 2
	* +	—	—	+	—	—	+	1	2 1
	* +	+	+	+	—	+	+	1	2 1
	+	—	—	—	—	—	—	1	2 1
	—	—	—	—	—	—	—	1	2 1
No	47	3	15	10	0	37	46		
Per cent	97 9	6 3	31 3	20 8	0	77 1	95 8		

\+ indicates transfer of unselected markers from F$^+$ parent

* These three recombinants had K(W1611)(L)

Recombinants selected on minimal medium supplemented with histidine

than those determining the K antigens behave like those in the cross just described No K antigen could be detected in 44 out of 48 recombinants, but four recombinants had the K antigen of the *Hfr* parent, probably indicating that recombination has occurred between the hypothetical *kap*$^+$ locus of the *F* strain and the locus for K antigen specificity of the *Hfr* strain

Regarding the transfer of determinants for the flagellar apparatus, no differences can be found between the two last-mentioned crosses Nearly all recombinants are motile, *i e* have the *fla*$^+$ marker of the recipient 77 1 and 74 1 per cent respectively belong to H16, and the remainder to H10

Brief mention shall be made of the following cross W3703 *Hfr* × D149 *F* which is another auxotrophic mutant of the test strain for K54 The cross was carried out on EMS medium without supplementation, and selection for both histidine and serine was therefore in action 48 recombinants were examined, all belonged to O25 and like the findings in the above-mentioned cross (Table 6) 43 out of 48 recombinants belonged to K54 while the remaining 5 carried the latent K antigen of W3703 = K(W1611)

Finally the outcome of a *crossing experiment designed to unveil a* latent locus for O specificity in a K12 strain shall be described It has been reported earlier that an O antigen cannot be detected in K12 strains Such strains are spontaneously agglutinable, the R antigen has not been examined

As apparent from the experiments described above in which the O antigen specificity follows the *histidine* marker, a hypothetical latent K12 O antigen might be found among recombinants from a cross between K12 *F* and a histidineless *F* strain equipped with an O antigen

The actual cross was carried out between W6 F^+ and W3478 *F* 48 recombinants examined with a regard to the O antigen were all spontaneously agglutinable (rough) Even though the recombinants were scored on unsupplemented minimal medium, this result would suggest that loci for O and R antigens are situated close to each other on the coli chromosome

In Table 8 a list is given of the different serotypes found in the crosses

TABLE 8

List of Seroformulas of Recombinants Found in Crosses Recorded in Tables 2 to 7

	Hfr or F+			F−			Recombinants		
	O	K	H	O	K	H	O	K	H
Table 2	—	—	H48	25	—	—	—	—	—
							—	—	*16*
							25	—	—
							25	—	*16*
Table 3	25	—	—	100	(B)	2	25	—	—
							25	—	*16*
							25	—	2
							100	(B)	2
							100	*(B)*	*16*
							100	*(B)*	—
Table 4	25		—	9	26(A)	—	25	—	—
							9	26(A)	—
Table 5	25		—	9	—	—	25	—	—
							25	—	*16*
							9	—	—
Table 6	25		—	6	54(L)	10	25	*54(L)*	16
							25	*54(L)*	*10*
							25	*54(L)*	—
							25	*(W1611)(L)*	*16*
							6	54(L)	10
							6	*54(L)*	*16*
Table 7	25		—	6	—	10	25	—	*16*
							25	—	*10*
							25	*(W1611)(L)*	*16*
							25	*(W1611)(L)*	*10*
							6	—	10

New hybrid serotypes are written in italics

DISCUSSION

Only few of the numerous papers on the genetics of *E. coli* deal with the fate of the antigens in sexual recombination experiments.

As already mentioned, *Furness* found part of the progeny motile in a cross between a motile K12 F^+ strain and an immotile F^- strain. The flagella of the motile progeny had the antigenic pattern of the F^+ parent. He concluded that this was in contrast to the Salmonellas, where, when motility is transduced to a non-flagellated strain, the latent genes determining the H antigens of the recipient strain are usually expressed. In the experiments reported here, motile progeny were found in a cross between two non-flagellated immotile strains in which cross as well as in other crosses the latent H antigen of an immotile strain appears. It will therefore be necessary to describe at least two loci determining the flagellar apparatus in *E. coli*. The situation is similar to the one found in *Salmonella* where one *fla* locus and two *H* loci are described, except that only one H phase and one *H* locus is known in *E. coli*. The *fla* and *H* loci in the crosses reported here are not closely linked, but in some of the crosses indisputable co-transfer of *his* and *H* loci suggests a linkage between these markers. *Makela* found a similar linkage between the *H1* locus and the *histidine* marker in *Salmonella abony*.

As regards the K antigens, the experiments reported might add a little to our scanty knowledge about the nature of the K antigens and especially about the interrelation between the single K antigen types (L, B or A). In crosses between *Hfr* W3703 (O25 : K (L) : H) and female strains with A or B antigens, the recombinants will be either O25 : K– or have O and K antigen as the female parent, depending on transfer or non-transfer of *histidine* locus. It looks as though markers for O and B and for O and A are closely linked. A cross between the same *Hfr* strain and a histidineless strain with L antigen—or a K– form of it—will give a few recombinants which in addition to the O antigen of the male parent also carry the latent K antigen of W3703, *i. e.* the L antigen which can be found in strain WG4 from which W3703 has been derived. If the cross involves the K^+(L) form as female parent, recombinants are formed which have the O antigen of the Hfr parent and the L antigen of the female parent, *i. e.* segregation of the genetic determinants for O and L antigen. It is therefore apparent that at least two loci are necessary to determine the coli L antigen, one which determines the antigenic specificity and one which—more vaguely—determines the presence or non-presence of the L antigen. It can be mentioned here that a recent report (*Ørskov & Ørskov* 1961 b) mentions a male coli strain which, when converting other coli strains to the male sex, at the same time transfers the genetic determinant for the L antigen. As this finding suggests episomal transfer of the L antigen determinant, it will be seen that many problems remain as regards the genetic determination of coli K antigens. Our knowledge about *E. coli* K antigens has many other gaps and it is to be hoped that genetical experiments in

collaboration with serological and chemical analysis may help to give us a more deep going understanding of their structure and of their mutual relationships

SUMMARY

A number of sexual recombination experiments using auxotrophic derivatives of *E coli* antigen test strains are described

Locus for histidine synthesis, for the O antigen and to a lesser degree for the H antigen are closely situated on the chromosome

The loci for the K antigen of the A and the B types are probably very close to the *his* and O antigen loci

No linkage can be found between locus for K antigen of the L type and the *his* locus

At least two loci operate to give an L+ form as it is found that the latent L antigen of an L parent can appear among the progeny

Two loci are necessary for the establishment of the H antigen, the *H* locus and the *fla* locus

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FROM THE UNIVERSITY OF BERGEN, SCHOOL OF MEDICINE, THE GADE INSTITUTE, DEPARTMENT OF MICROBIOLOGY, BERGEN, NORWAY

IMMUNOCHEMICAL STUDIES ON POLYSACCHARIDE A OF STAPHYLOCOCCUS AUREUS

1 *Purification on DEAE Cellulose Columns*

By

GUNNAR HAUKENES

Various methods of extracting antigenic material from a strain of *Staphylococcus aureus* have been examined (2) The technique preferred was *disruption of the bacteria in a bacteria press followed* by extraction with a buffer in the cold A serologically highly active polysaccharide was obtained by precipitating the extract with ethyl alcohol after removal of acid precipitates This polysacccharide has been studied further immunochemically, and the results of these studies will be reported in a series of publications Some chemical and serological data on the purified polysaccharide have been given in a *preliminary note (1)*

The present paper deals with separation of the polysaccharide from contaminating substances by chromatography on DEAE cellulose columns It will be shown later that the serological reactivity of our crude polysaccharide undoubtedly is identical to that of polysaccharide A, described in pathogenic staphylococci by *Julianelle & Wieghard* (1934) For convenience, the designation polysaccharide A has been *adopted in the present paper Since the publications of the above-men*tioned authors (7, 8, 9, 17), no report dealing with the immunochemical properties of polysaccharide A has appeared

METHODS

Unfortunately the strain used by *Julianelle & Wieghard* (7) and others (15 16) seems to have been lost It has been requested from several type culture collections and laboratories without result Type strain 1503 (10) has been used in the present experiments Some serological data on this strain have been given in previous papers (2 3)

Rabbit immune sera were produced by intravenous injections of formalin killed organisms as described by *Oeding* (1957) Most sera against strain 1503 were found to yield good precipitation reactions against polysaccharide A on the ring test and agar precipitation

Ring test precipitation in capillary tubes was performed with dilutions of antigen in saline layered over undiluted immune serum It was found important to use strong antisera for ring test precipitation avoiding negative reactions by antigen excess Thus a strong antiserum reacted with concentrations of *polysaccharide A* ranging from 25 µg to 0 5 g per 100 ml

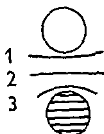


Fig 1

Schemat c drawing of the major lines given by strain 1503 and its antiserum

○ Suspension of crushed bacteria

⊜ Antiserum undiluted

1 Antigen *n* line

2 Antigen A (Jensen) line

3 Polysaccharide A line

Agar precipitation Applying *Ouchterlony s* method (13) six circular peripheral an l one central well were cut in the agar The distance between the wells was 10 mm The techn que has been described more detailed in earl er publications

also important that readings are made at least once daily during the first days since the polysaccharide A line may migrate into the serum basin and disappear

the antigen isolated from strain Cowan I and purified as described by *Jensen* (1959) Unfortunately the designati n antigen A may easily be confused with I lysaccharide A We have therefore in this and forthcoming papers use l the designation antigen A (Jensen) In add tion to these three lines two or three other l nes are more or less c nstantly found These lines have not been identified yet but they seem to represent group antigens

The pH was measured in a pH Meter 22 Radiometer Copenhagen with glass electro le type G 200 A When less exact values sufficed Merck s Special Indicator papers were used

The ptical density at 280 mμ was measured in a Unicam SP 500 spectrophotometer with 1 0 cm cells

Dry weight estimation The substance to be examined was lyophilized and then drie l under reduced pressure in a desiccator for 48 hours at room temperature Weighing was perf rmed with a Mettler analytical balance type F 5 Since the preparations were to l e used for serological reactions the normal procedure of drying in an oven at 105° C was omitted Compar ng the tw

s metimes gave the same sometimes

Dialysis was performed in cell

saccharide A passe l the cellophane

Sephadex G 25 columns for gel

fr m the manufacturer (Pharmaci

5 0 × 45 cm were used depend ng

total l ed volume ought to be at least thrice the volume of the sample to obtain complete separation of salts from c lloids Polysaccharide A behaved as a colloid

not being retained by the gel. A representative example of separation of polysaccharide A from salts on a 5.0 × 45 cm Sephadex G 25 column shall be given. Two hundred ml of a salt containing polysaccharide A solution was applied to the column and eluted with distilled water. First, 235 ml of water passed the column. Then polysaccharide A appeared in the next 300 ml. No salts were found in these fractions. After the passage of an additional 150 ml of water, the salts appeared. When only traces of salts were present in the sample, chlorides or sulphates were added to enable detection of salt containing fractions. Chlorides were estimated as indicated below, and sulphates were easily detected by precipitation with barium hydroxide.

Diethylaminoethyl (DEAE) cellulose columns were prepared from Solka Floc by the method of *Peterson & Sober* (1956). Columns of 1.0 × 27 cm were employed for amounts of polysaccharide not exceeding 300 mg. For greater amounts of polysaccharide 2.5 × 30–40 cm columns were preferred to avoid overloading the resin. The columns were packed to a pressure of about 50 cm water, and equilibrated with a 0.02M phosphate buffer of pH 7.4. Since the procedures were carried out at room temperature under unsterile conditions toluene was added to the sample and to the eluants to avoid microbial contamination. When gradient elution was to be established, two flat-bottomed, cylindrical flasks of equal size were connected by a rubber tube. The tube was filled with the fluid and worked as a siphon to maintain the same fluid level in both flasks. In this way the concentration of salts in the eluant increased evenly. A magnetic stirrer assured thorough mixing. Effluent fractions of 10 ml were collected and the flow rate was regulated to about 15 ml per hour. The optical density of each fraction was measured at 280 mμ. This wavelenght was preferred since toluene interferred greatly with the recording in the region of 260 mμ but not at 280 mμ.

ECTEOLA-SF cellulose was prepared from Solka Floc according to the method of *Peterson & Sober* (1956). The column was run by the technique described for DEAE cellulose.

Chlorides were estimated by titration with silver nitrate, using potassium chromate as an indicator.

Unless otherwise stated, all operations were carried out in the cold.

EXPERIMENTAL

The crude polysaccharide material was prepared as described before (2) with two modifications. First, the crushed bacteria were extracted with acetate buffer of pH 4.2 and pH 7.0 alternately. More polysaccharide A seemed to be extracted at pH 7.0, and the amount increased with the lenght of time. The nature of the extraction process has been studied further, and the results will be published later. The second modification comprised precipitation of the crude polysaccharide solution with alkali. When the pH was increased to 11.5–12.0 by the addition of N sodium hydroxide, a flocculent precipitate was formed. This was immediately removed by centrifugation, and the pH adjusted to neutrality. The precipitate was serologically inactive on the ring test and agar precipitation, and was discarded.

Preliminary experiments with DEAE cellulose columns had shown that polysaccharide A was eluted between effluent molarities of potassium chloride of 0.10 and 0.30, when a gradient up to a molarity of 0.40 was applied. As a routine, therefore, elution was started with a solution of 0.08M potassium chloride. At this chloride concentration no polysaccharide A was eluted. Elution was continued until the fractions showed no absorption at 280 mμ and a negative ring test. Gradient elution was then established affording an even increase in chloride

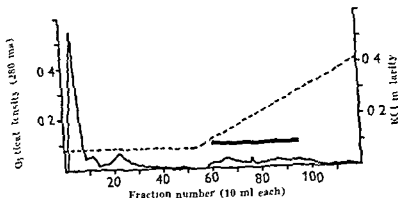


Fig 2

Chromatography of polysaccharide from strain 1503 on DEAE cellulose

——— 280 mμ

- - - - Effluent molarity

▬▬ Polysaccharide A present in the eluate on ring test

concentration to 0 40 in the course of 800 ml All eluants contained 0 02M phosphate buffer maintaining the pH at 7 4 throughout the experiment The ionic strength was therefore somewhat greater than indicated by the potassium chloride molarity Polysaccharide A was easily detected in the effluent by the ring test, yielding a firm and sharp disc of precipitate, even at high dilutions

In the present experiment 100 g of wet crushed bacteria was extracted as indicated above for seven days The yield of crude polysaccharide was 360 mg Three hundred mg of this polysaccharide dissolved in phosphate buffer was applied to a 1 0 × 27 cm column containing 2 2 g of DEAE cellulose The resin had previously been equilibrated with the 0 02M phosphate buffer at pH 7 4 Effluent fractions of 10 ml were collected and elution was achieved as indicated above The first peak shown in Figure 2 represented material passing straight through the column The ring test was positive on these fractions being maximal in fractions 4 and 5 The disc of precipitate formed however was less firm and sharp than that given by polysaccharide A On agar precipitation an antigen A (Jensen) line (see under Methods) was given by fractions 4 to 10 most pronounced in fractions 4 5 and 6 No polysaccharide A line was found in these fractions At a potassium chloride molarity of 0 10 i e fraction number 57, polysaccharide A appeared indicated by the characteristic ring test precipitation Fractions giving a strongly positive ring test from number 60 to 95 were collected These fractions also exhibited a slight increase in optical density at 280 mμ The salts were removed by dialysis" on a Sephadex G 25 column and the polysaccharide was then lyophilized

On rechromatography (Figure 3) of the lyophilized portion by the

not being retained by the gel A representative example of separation of polysaccharide A from salts on a 5 0 × 45 cm Sephadex G 25 column shall be given Two hundred ml of a salt-containing polysaccharide A solution was applied to the column and eluted with distilled water First, 235 ml of water passed the column Then, polysaccharide A appeared in the next 300 ml No salts were found in these fractions After the passage of an additional 150 ml of water, the salts appeared When only traces of salts were present in the sample chlorides or sulphates were added to enable detection of salt containing fractions Chlorides were estimated as indicated below, *and sulphates were easily detected by precipitation with barium hydroxide*

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DISCUSSION

Separation of polysaccharide A on DEAE cellulose columns resulted in a preparation which reacted to higher titres on ring test precipitation than the original crude preparation On the first chromatographic run antigen A (Jensen) and some ultraviolet light (U V) absorbing material were removed Both substances were found in the first fractions under the conditions employed being eluted at a lower molarity than polysaccharide A Antigen A (Jensen) is supposed to be a polysaccharide (6) but very few chemical data have been published In the first chromatogram fraction number 3 produced no antigen A (Jensen) line on agar precipitation while fraction number 6 produced a strong line The extinctions of the two fractions were 0 418 and 0 133 respectively The U V absorbing material in our experiment therefore does not seem to be identical with antigen A (Jensen), or at least some of this material must be different from antigen A (Jensen) The ring test reactions however corresponded well to the agar precipitation having a common maximum in fractions 4 and 5

The fractions containing polysaccharide A showed a slight absorption of ultraviolet light Whether this is caused by the polysaccharide itself or by impurities is not clear and can only be settled when all impurities have been removed

The purified polysaccharide preparation after rechromatography still contained impurities of antigenic material The contaminating [illegible]

Chromatography on an ECTEOLA cellulose column did not seem to offer advantages over DEAE cellulose

Desalting by gel filtration was found to be effective and time saving and was preferred to dialysis

Other methods of purification should be attempted to remove the last impurities The introduction of other strains may render the purification more easy Addition of alkali to precipitate inactive material may cause degradation of the polysaccharide and it has to be settled whether this purification step can be omitted

SUMMARY

Crude polysaccharide material from *Staph aureus* strain 1503 has been purified by chromatography on DEAE cellulose columns Polysaccharide A separated well from impurities of antigen A (Jensen) and some U V absorbing material Agar precipitation experiments however showed that the preparation still contained small amounts of impurities which calls for additional purification methods

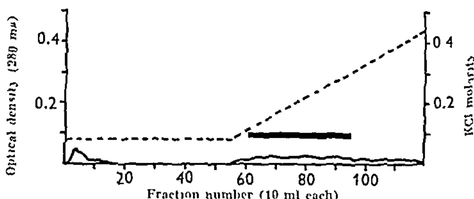


Fig 3

Rechromatography of polysaccharide from strain 1503 on DEAE cellulose

——— 280 mμ

- - - - Effluent molarity

▬▬ Polysaccharide A present in the eluate on ring test

same technique, a low peak again appeared in the first fractions Antigen A (Jensen) was not found in these fractions on agar precipitation Polysaccharide A was, as before, eluted in fractions 60 to 95 The collected fractions were desalted, twice, on Sephadex G-25 columns and lyophilized

The recovery of polysaccharide A after chromatography at first seemed to be low Later, it was discovered that a considerable amount of material was lost during freeze-drying The actual figures for recovery, therefore, cannot be given

Crude and purified polysaccharide preparations were compared by the ring test and agar precipitation The ring test titre of the crude polysaccharide was 1 10⁶, while the purified polysaccharide reacted to 1.4×10^6 on serial dilution of the preparations in saline against undiluted antiserum Both preparations produced strong polysaccharide A lines on agar precipitation, and the crude polysaccharide in addition gave a distinct antigen A (Jensen) line The purified sample also produced an additional line midway between the basins The line was very faint and was detected when polysaccharide concentrations of 0 1 g per 100 ml or higher were employed The identity of the line was not settled, but most probably it represented a residue of antigen A (Jensen)

Purification was also attempted on an ECTEOLA column using the same technique Polysaccharide A was eluted at the same molarities as with DEAE cellulose, but the polysaccharide A fractions showed higher extinction values at 280 mμ

The crude and purified polysaccharide preparations have been examined chemically, and the results will be published later

FROM THE UNIVERSITY OF BERGEN, SCHOOL OF MEDICINE, THE GADE INSTITUTE DEPARTMENT OF MICROBIOLOGY, BERGEN, NORWAY

IMMUNOCHEMICAL STUDIES ON POLYSACCHARIDE A OF STAPHYLOCOCCUS AUREUS

2 *Further Studies on Purification Methods*

BY

GUNNAR HAUKENES

Received 22 xi 61

Preceding papers (2, 3) have reported the preparation of the group-specific polysaccharide A (6) from the coagulase-positive staphylococcal type strain 1503 (8) A crude polysaccharide material (3) was purified by chromatography on DEAE cellulose columns Polysaccharide A separated well from the impurities, antigen A (Jensen) (5) and ultraviolet light (U V) absorbing material, on the cellulose columns The method failed, however, to yield a completely pure preparation as agar

[illegible]

.. chromatography
All [illegible]
were [illegible]
of sp[illegible]
shoul[illegible] from impurities of antigen A (Jensen) Search was made accordingly for a strain of *Staphylococcus aureus* possessing an easily extractable polysaccharide A and little or no antigen A (Jensen) The 13 type strains employed by *Haukenes & Oeding* (4), were examined serologically, and strain Wood 46 proved to fulfil these demands Of the strains examined, strain Wood 46 produced by far the most potent serum against polysaccharide A and the strongest polysaccharide A line on agar precipitation Few other precipitation lines were detected, and the strain seemed to lack antigen A (Jensen) entirely.

In the present work polysaccharide A has been prepared from the strain Wood 46, and various purification methods have been attempted

METHODS

Strain Wood 46 is used throughout the world as it is a good producer of alpha toxin while it produces no beta toxin The colonies produced on our blood agar and nutrient agar were white and comparatively small The strain lacked the clumping factor but coagulated citrated plasma readily On serological typing the strain agglutinated strongly in a factor serum (4), and the present author has found a weak agglutinating factor related to the *a* antigen (unpublished) When

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All of the normal human sera examined by *Jensen* (1958) 500 in all were found to contain antibodies to antigen A (Jensen) It is therefore of special importance in immunological research that polysaccharide A should be free from impurities of antigen A (Jensen) Search was made accordingly for a strain of *Staphylococcus aureus* possessing an easily extractable polysaccharide A and little or no antigen A (Jensen) The 13 type strains employed by *Haukenes & Oeding* (4) were examined serologically and strain Wood 46 proved to fulfil these demands Of the strains examined strain Wood 46 produced [illegible]

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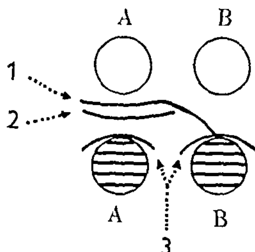


Fig 1

Schematic drawing of the major lines given by strain Wood 46 and its antiserum (B) Strain F 21 and its antiserum (A) serve as a reference system for the antigen *i* and antigen A (Jensen) lines

○	Suspensions of crushed bacteria
⊜	Antisera diluted 1 2 in saline
1	Antigen *i* line
2	Antigen A (Jensen) line
3	Polysaccharide A lines

examined with the routine set of 24 phages the strain revealed the patterns 7 and $42D_w$ at Routine Test Dilution (RTD) and 1000 times RTD respectively Strain 1503 has been presented in (3)

Various staphylococcal rabbit antisera were used in the present experiments Strain Wood 46 antiserum was found to be well suited for agar precipitation Owing to its high antibody content a strong polysaccharide A line was produced and negative reactions due to antigen excess were never recorded Strain 1503 antiserum was used for ring tests Strain F 21 antiserum (8) was used for demonstrating the antigen *i* line (*Oeding* & *Haukenes* unpublished) and the antigen A (Jensen) line

Agar precipitation The principal precipitation lines given by strain Wood 46 have been drawn in Figure 1 Owing to a low content of *i* antibodies in strain Wood 46 antiserum strain F 21 antiserum was used to demonstrate the antigen *i* line of strain Wood 46 One or two additional unidentified lines have been found but no antigen A (Jensen) line

Sephadex G 25 columns have been used for desalting instead of dialysis On gel filtration polysaccharide A from strain Wood 46 behaved like polysaccharide A from strain 1503 separating well from the salts

The preparation of DEAE cellulose and the elution techniques have been described in (2)

Charcoal column Charcoal (Carbo activatus siccus Merck) was suspended in distilled water and poured as a slurry into a column of 1 0 cm diameter to a height of 7 cm The sample was then added and various eluants were tried

Amberlite IR 120 column Amberlite IR 120 resin 8 per cent cross linkage was sieved to 100–230 mesh particle size The exchanger was suspended in 4N hydrochloric acid after two cycles through the Na form and transferred to a column of 1 0×27 cm

Dowex 1 columns were prepared from Dowex 1 (Bio Rad) 8 per cent cross linkage 200–400 mesh particle size and in the chloride form The resin was suspended repeatedly in water and decanted to remove the finer particles The suspension was

poured into a column and 3M s lium formate was ad led until all chl rides were rem e l Then the res n was washed through with water whereupon five column volumes f 80 per cent form c acid were] assed through the column Finally the c lumn was washe l with water until the eluate contained no acid Elution was ach eved with ammonium f rmate buffer of pH 5 0 with toluene a l led The molarities of the effluent fractions were calculated from the effluent volumes

All columns were run at room temperature (16-22° C) Distillat n under reduce l pr ssure was carr e l ut at a temperature of °7 28° (All other operations were carr ed out in the cold

The techniques of immun zation ring test precipitation pH measurement an l dry weight est mation and the types of appartus used have been described in (2) and (3)

EXPERIMENTAL

Preliminary Experiments

The crude polysaccharide used for these experiments was prepared as described in the preceding paper (2) The ring test titre of the crude polysaccharide was 1 2×10 On agar precipitation a strong polysaccharide A line was found but no additional lines

DEAE cellulose columns

Experiment 1 About 100 mg of crude polysaccharide material from strain Wood 46 was placed upon a 1 0×26 cm column containing 2 0 g of DEAE cellulose The column was first eluted with 0 02M phosphate buffer of pH 7 4 and then with increasing concentrations of potassium chloride as described previously (2) Polysaccharide A Wood 46 like polysaccharide A 1503 was retained by the resin and appeared in the eluate at a potassium chloride concentration of about 0 13M The technique used for polysaccharide A 1503 i e removal of material eluted at 0 08M potassium chloride before gradient elution was therefore adopted in the following experiments

Experiment 2 Polysaccharide A Wood 46 recovered from experiment 1 was applied to a new cellulose column Elution was carried out as shown in Figure 2 Fractions containing polysaccharide A displayed some optical density at 280 mμ Earlier experiments (2) have shown that polysaccharide A 1503 absorbed little or no light in the region of 280 mμ It was therefore important to see whether polysaccharide A Wood 46 could be separated from the U V absorbing material Chromatography on Amberlite IR 120 was first attempted (described after the cellulose experiments) and then on DEAE cellulose at various pH values Material recovered from one experiment was used in the next

Experiment 3 The DEAE cellulose column was run as before with the exception that the pH was maintained at 4 6 by a 0 02M phosphate buffer Figure 3 shows the extinction values at 280 mμ and the polysaccharide A containing fractions Only fractions collected during gradient elution have been depicted in this figure It is obvious from Figure 3 that separation at pH 4 6 offers no advantage over the routine method at pH 7 4

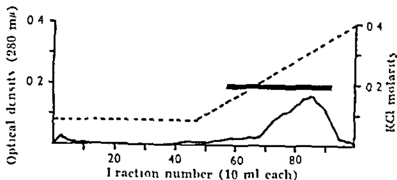


Fig. 2

Chromatography of polysaccharide from strain Wood 46 on DEAE cellulose at pH 7.4

—— 280 mμ

...... Effluent molarity

▬ Polysaccharide A present in the eluate on ring test

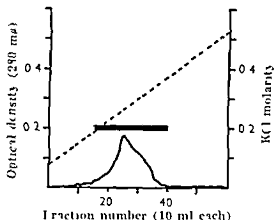


Fig. 3

Chromatography of polysaccharide from strain Wood 46 on DEAE cellulose at pH 4.65

—— 280 mμ

.... Effluent molarity

▬ Polysaccharide A present in the eluate on ring test

Experiment 4. The column was run at pH 9.0. The result was much the same as in experiments 2 and 3, and details will not be given.

Experiment 5. The pH was increased to 11.0 by a 0.06M phosphate buffer (Figure 4). Comparison of ring test titres and optical densities revealed that fraction number 18 reacted at a higher titre than fraction 22, while the extinction value of fraction 22 was 4 to 5 times higher than that of fraction 18. This suggested that at least the major part of the U.V. absorbing material was not identical to polysaccharide A, with reservation for alkaline degradation. This method, however, was not very practical for the preparation of polysaccharide A.

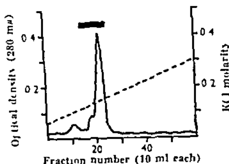


Fig 4

Chromatography of polysaccharide from strain Wood 46 on DEAE cellulose at pH 11 0

—— 280 mμ

- - - - Effluent molarity

▬ Polysaccharide A present in the eluate on ring test

Amberlite IR 120 column Polysaccharide material recovered from the DEAE cellulose experiment 2 was applied to a column of Amberlite IR-120 in the hydrogen form The fractions were examined, as before, by the ring test and for ultraviolet absorption Polysaccharide A together with U V absorbing material went straight through the column on elution with water These fractions were collected and used in the DEAE cellulose experiment 3

Charcoal column Crude polysaccharide from strain Wood 46 was adsorbed to charcoal The column was eluted with 40 per cent alcohol, 10 per cent acetic acid, and 0 3M potassium chloride successively Neither polysaccharide A nor U V absorbing material appeared in the eluate

Dowex 1 columns The strong anion exchanger, Dowex 1, has been widely used for separation of nucleotides (1), and was therefore tried In a preliminary experiment 5 mg of crude polysaccharide from strain Wood 46 was applied to a 1 0×24 cm column of Dowex-1 in the formate form Elution was started with water, whereupon gradient elution was established affording an even increase of ammonium formate concentration to 1 0M in the course of 1050 ml Thereafter the concentration of formate in the eluant was increased to 2 5M The pH of the ammonium formate buffer was 5 0 Polysaccharide A was eluted between the molarities of 0 4 and 0 7 in this experiment These fractions showed no absorption at 280 mμ Ultraviolet light absorbing material was eluted between 1 0 and 2 5M of ammonium formate

In another preliminary experiment stepwise increase in molarity was tried Polysaccharide A material recovered from the DEAE cellulose experiment 5 originally 100 mg crude polysaccharide which had passed several columns, was applied to the column Elution was started with

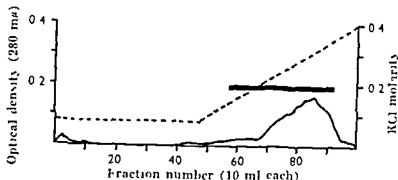


Fig 2.

Chromatography of polysaccharide from strain Wood 46 on DEAE cellulose at pH 7 4

—— 280 mμ

----- Effluent molarity

▬ Polysaccharide A present in the eluate on ring test

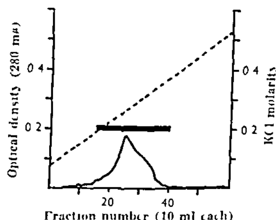


Fig 3

Chromatography of polysaccharide from strain Wood 46 on DEAE cellulose at pH 4 65

—— 280 mμ

----- Effluent molarity

▬ Polysaccharide A present in the eluate on ring test

Experiment 4 The column was run at pH 9 0 The result was much the same as in experiments 2 and 3, and details will not be given

Experiment 5 The pH was increased to 11 0 by a 0 06M phosphate buffer (Figure 4) Comparison of ring test titres and optical densities revealed that fraction number 18 reacted at a higher titre than fraction 22, while the extinction value of fraction 22 was 4 to 5 times higher than that of fraction 18 This suggested that at least the major part of the U V absorbing material was not identical to polysaccharide A, with reservation for alkaline degradation This method, however, was not very practical for the preparation of polysaccharide A

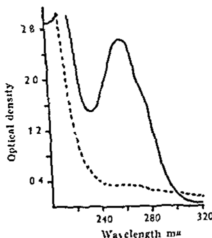


Fig 6

Ultraviolet absorption spectra of two polysaccharide A Wood 46 preparations in 0 2 per cent aqueous solution

—— Crude polysaccharide A

- - - - Polysaccharide A purified on Dowex-1

Preparation of Greater Amounts of Polysaccharide A

Chromatography on Dowex-1 seemed to provide satisfactory resolution of polysaccharide A and impurities This method in addition to separation on DEAE cellulose, was employed to prepare polysaccharide A from strain Wood 46 in amounts sufficient for chemical and immunological investigations

The bacteria were harvested from 24 hour old agar cultures by scraping the surfaces The total wet weight was 175 g The crushed cells were extracted at 0° C with a phosphate buffer of pH 6 5 Extracts were obtained at intervals by centrifuging, and extraction was continued for 24 days The amount of polysaccharide A in the extracts was at least 500 mg calculated according to a ring test titre of $1 \cdot 4 \times 10^{6}$ on the purified material Precipitation with acid and alcohol was carried out as before, but precipitation with alkali at pH 11 0 was omitted During preparation of the crude polysaccharide the pH was never brought above neutrality

Chromatography on DEAE cellulose was carried out by the routine technique The size of the column was 2 5×28 cm

Polysaccharide material separated on DEAE cellulose was placed upon a 2 5×28 cm column of Dowex-1 in the formate form Elution was achieved with ammonium formate buffer as in the first preliminary experiment with Dowex-1 Polysaccharide A appeared in the eluate at an ammonium formate molarity of about 0 1 At this point

350 ml of 0 45M ammonium formate, *i e* a molarity which is a little higher than the molarity at which polysaccharide A appeared in the eluate in the previous experiment Elution was then continued with 200 ml of 0 8M ammonium formate and finished with 2 5M Toluene was not added in this experiment, and the extinction values were recorded at both 260 and 280 mμ

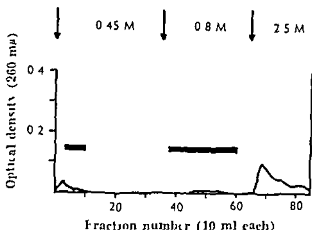


Fig 5

Chromatography of polysaccharide from strain Wood 46 on Dowex 1 by stepwise increase of ammonium formate molarity

—— *260 mμ*

▬ Polysaccharide A present in the eluate on ring test

Figure 5 shows that some U V absorbing material went straight through the column together with small amounts of polysaccharide A, while the greater part of the U V absorbing material appeared on elution with 2 5M The bulk of the polysaccharide A was eluted between the molarities of 0 45 and 0 8, well separated from the fractions showing ultraviolet absorption The U V absorbing material showed higher extinction values at 260 mμ than at 280 mμ, and only the former values have been depicted in Figure 5

For future work the following elution technique will be preferred. First elution with distilled water, followed by gradient elution from 0 to 1 0M, and finally elution with 2 5M ammonium formate

The polysaccharide A material obtained before and that obtained after chromatography on Dowex-1 in the first preliminary experiment, were compared by the ring test, and the titres were $1\ 2\times10^{6}$ and $1\ 4\times10^{6}$ respectively The ultraviolet absorption spectra of the preparations were recorded (Figure 6), and showed that the purified sample displayed very low extinction values and no peak when examined in aqueous solution

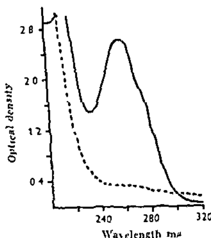


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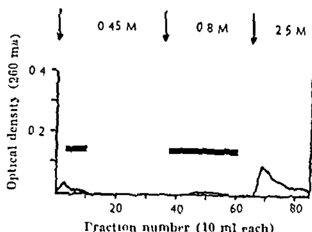


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—— 260 mμ

▬ Polysaccharide A present in the eluate on ring test

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and this procedure can, therefore, safely and with advantage be omitted. By the methods employed, examination of polysaccharide A Wood 46 revealed no impurities of antigenic material, and this preparation will be subjected to chemical and immunological investigations.

The ring test titre of polysaccharide A Wood 46, like that of the purified sample described in the preceding paper (2), was 1:4×10⁶. Such high titres are often given by bacterial polysaccharides. *Julianelle & Wieghard* (7) examined polysaccharide A preparations from different strains of pathogenic staphylococci and, using a somewhat different precipitation technique, obtained serological reactivity in dilutions up to 1:6–8×10⁶.

Chromatography on Amberlite IR-120 in the hydrogen form afforded no separation of polysaccharide A from the contaminants, and elution of material adsorbed to charcoal was not successful. Neither of these methods were, however, examined more extensively, as the Dowex-1 exchanger appeared to give satisfactory resolution of the components. In the first preliminary experiment all impurities of the crude polysaccharide A Wood 46 seemed to be removed by chromatography on Dowex 1. Whether separation on DEAE cellulose can be omitted, has not been settled since serologically inactive material not absorbing ultraviolet light may be removed without being detected. Hence, both procedures have to be carried out until further data or other methods are available. The amount of polysaccharide A applied to the Dowex 1 column in the latter experiment was obviously too high, since elution had to be continued with 1.0 M ammonium formate. This may involve risk of overlapping into the group of fractions absorbing ultraviolet light.

SUMMARY AND CONCLUSIONS

Methods for purifying polysaccharide A have been studied further, including adsorption to charcoal and chromatography on the cation exchanger Amberlite IR 120 and the anion exchanger Dowex 1. The latter was found to separate polysaccharide A well from U.V. absorbing material. The introduction of strain Wood 46 abolished the difficulties involved in the removal of antigen A (Jensen).

Based upon these and previous investigations (2) the following method for the preparation of polysaccharide A is suggested:

1. Extraction of crushed cells of strain Wood 46 by a buffer of pH 6.5 at 0° C.

2. Crude polysaccharide is obtained from the extracts by removal of acid precipitates at pH 4.2 and repeated alcohol pre[illegible]

3. P[illegible]

by re[illegible]

lectir[illegible]

tion on gradient elution [illegible] and characteristic ring test precipita

some fractions showed some optical density at 280 mμ. These and the subsequent ten fractions, which showed no absorption, were combined before collecting the polysaccharide A containing fractions. The amount of polysaccharide A in the combined fractions was less than 1 mg, and the loss by this procedure was thus negligible.

Polysaccharide A was still found in the eluate when a molarity of 1.0 was reached. In order to avoid contamination with U.V. absorbing material, the ammonium formate concentration was not raised above this value until most of the polysaccharide had been recovered, *i.e.* until the ring test had become only weakly positive. Apart from the above-mentioned first fractions which were combined, none of the fractions containing polysaccharide A showed any absorption at 280 mμ. On elution with 2.5M ammonium formate, U.V. absorbing material appeared. Further elution with 5M ammonium formate was attempted, but no more material was obtained.

The fractions containing polysaccharide A were combined, and the volume reduced by distillation under reduced pressure. The polysaccharide was then obtained by alcohol precipitation. After desalting on Sephadex G-25 the material was lyophilized and dried in a desiccator to constant weight. The yield was 456 mg of polysaccharide A, and this batch was used for all future experiments with polysaccharide A Wood 46.

The ring test titre of the purified polysaccharide A Wood 46 was 1:4×10^6. The preparation in varying concentrations was examined by agar precipitation against strains Wood 46, F 21, and 1503 antisera. The highest dilution of polysaccharide A producing the specific line, was 1:5×10^5. Concentrations of the antigen up to 0.5 g per 100 ml were employed, but no additional lines were found. Polysaccharide A lines given by preparations from strain Wood 46 and strain 1503 showed a "reaction of identity". The combined fractions eluted at the appearance of polysaccharide A in the eluate on the Dowex-1 column, produced a weaker polysaccharide A line and no additional lines.

A sample of purified polysaccharide A Wood 46, 0.1 g per 100 ml, was treated with alkali to pH 11.0, but no precipitation ensued.

DISCUSSION

Strain Wood 46 was found to be well suited for the preparation of polysaccharide A. The antigen was easily extracted, and a relatively large yield was obtained. Of special importance to the purification was the lack of antigen A (Jensen). The crude polysaccharide Wood 46 was contaminated with U.V. absorbing material, but no antigenic components, when examined by agar precipitation. Chromatography on DEAE cellulose at various pH values failed to resolve the components, whereas this was achieved by the Dowex-1 anion exchanger. No precipitation followed the addition of alkali to the purified polysaccharide,

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BRIEF REPORT

METHOD FOR ISOLATING PIG GRANULOSA CELL AGGREGATES IN AMOUNTS ALLOWING BIOCHEMICAL INVESTIGATION OF STEROID HORMONE SYNTHESIS IN VITRO

By *Lars Bjersing*

The site and mode of formation of the ovarian hormones has long challenged research A difficulty obstructing the approach of the problem is the lack of methods for isolating and studying separate tissue elements of the ovary *Zondek & Aschheim* (1926) (7) isolated granulosa cells in a couple of mature human follicles by carefully scraping them from the theca layer By application of suction to the ... *estman* (1929) (6) succeeded in re- ... ars to the theca In 1959 *Falck* (3) ... a cells from rat ovaries for micro- ... to above the methods aimed at securing small amounts of tissue sufficient to evaluate the hormone production by biological indicators

Biochemical methods including radioactive isotope techniques have now enabled assessment of ...

... of environmental factors and gonadotropic hormones on the synthetic activity ...

To check the ...

... with a sharp, pointed instrument about 1 5 mm in diameter With a blunt pincette the follicle was compressed ...

... centrifuged In successful cases 50 to 100 mg tissue was obtained from a pair of ovaries ... d in sublimate- ... an (4) and ex- ... of treatment up ... Then, by gentle

Received 13 ix 62 *from the University Department of Pathology Allmänna sjukhuset Malmö Sweden*

4 Further purification of the material by separation on a Dowex 1 column applying gradient elution with ammonium formate buffer of pH 5 0

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FROM THE DEPARTMENT OF FORENSIC MEDICINE, UNIVERSITY OF HELSINKI
HEAD: PROFESSOR UNTO UOTILA

THE QUANTITY OF COBALT NEEDED TO PRODUCE POLYCYTHEMIA IN THE RAT

By

JOUKO SAIKKONEN

Received 20.xi.61

In 1929 *Waltner & Waltner* (11) observed, that polycythaemia is produced by the addition of 0.5 per cent pulverised metallic cobalt to the diet of normal rats or when 0.01 to 0.1 gm of cobalt nitrate is injected subcutaneously. Since this discovery, the effect of cobalt has been tested in other mammals and in various situations.

No doubt, this element seems to accelerate erythropoiesis. After daily cobalt administration reticulocytes and erythroblasts are found in the peripheral blood (2), and the bone marrow shows hyperplasia of the erythropoietic elements (5). An increase is seen in the blood volume as well as in the total erythrocyte mass and count, while the plasma volume has decreased (9). A significant polycythaemic reaction, as indicated by an increase in the erythrocyte count and haemoglobin value, is usually seen after 2 to 3 weeks of cobalt treatment.

The doses used by *Waltner & Waltner* were relatively high as [illegible] studies *Stanley* and co- [illegible] ent amounts of cobalt on [illegible] cobalt chloride ($CoCl_2$ $6H_2O$) was administered orally and subcutaneously. Daily injections of 2.5 mg per kg of body weight over a period of 6 weeks resulted in an increase of more than 30 per cent in the number of erythrocytes and in the amount of haemoglobin. To produce a similar effect by oral administration within the same period of time, 40 mg per kg of body weight was required. These dosages were without significant toxic effects. In rats receiving subcutaneous injections of 10 mg of cobalt chloride per kg of body weight daily, the weight loss averaged 20 per cent by the end of 6 weeks. Rats receiving 40 mg/kg subcutaneously did not survive beyond 8 days. In the experiments of *Gessert & Phillips* (4) cobalt chloride was incorporated in the diet of rats in the amounts of 0, 10, 20, 40, 70, and 100 p.p.m. of added cobalt. The authors claimed that the ratio of the increase in haemoglobin concentration in each polycythaemic group to the normal increase in 14 weeks was found to be directly proportional to the logarithm of the concentration (expressed in p.p.m.) of added dietary cobalt.

warming it was possible to remove the lump of paraffin containing the cells Of 30 consecutively prepared pairs of ovaries acceptable material was obtained from 25

Microscopically numerous closely packed granulosa membranes were seen Here and there single or small groups of red blood cells were found and occasionally a few theca cells and/or blood vessels but this admixture was insignificant A more detailed report will be published

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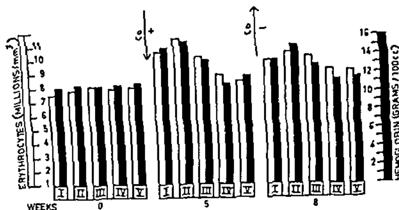


Fig 1

Group I 0 4 mg of Co per kg of body weight daily
II 0 2 mg
III 0 1 mg
IV 0 05 mg "
V 1 ml of 0 9 per cent saline daily

For further explanation see text

crease of 4 per cent is seen both in the erythrocyte count and in the haemoglobin

3 weeks after treatment had been discontinued the blood values in groups I, II, and III decreased although they were still above the original level and values of the control animals In group I haemoglobin exceeded the original value by 25 per cent and the erythrocyte count by 33 per cent The corresponding figures in group II were 33 per cent and 37 per cent and in group III 18 per cent and 28 per cent respectively Group IV no longer showed a significant difference from the controls

In all groups the erythrocyte count had increased more than the haemoglobin value This was especially the case in group IV The weight of the animals increased steadily throughout the experimental period by about 10 per cent in each group

DISCUSSION

The present experiment shows that cobalt administered to the adult male albino rat subcutaneously in a dose of 0 05 mg of cobalt daily per kilogram of body weight over a period of 5 weeks causes a slight increase in the number of erythrocytes whereas the haemoglobin value is not affected When the dose is doubled, *i e* to 0 1 mg per kilogram of body weight, a significant polycythaemia is produced The polycythaemic reaction after a dose of 0 2 mg/kg is somewhat stronger, but no further erythropoietic effect can be obtained apparently by increasing the dose to 0 4 mg/kg This is in good agreement with earlier reports (8) that doses of 5 mg/kg produced no further increase in

Besides its erythropoietic effects, cobalt has many toxic properties and causes. disturbance of growth (3), weight loss (10), lack of appetite and vomiting (6), development of goiter (1), porphyrinuria and adrenal hyperplasia (8) *Rothlin* and associates (7) studied the toxicity of different cobal salts in animal and human experiments Cobalt chloride and cobalt sulphate were shown to be about twice as toxic as cobalt glutaminate LD 50 in the mouse in 25 hours is 0 275 mg per gram of body weight when cobalt is administered as glutaminate per os and only 0 135 mg when cobalt chloride is given In human therapy these authors recommend a dose of 0 5 mg of cobalt per kilogram of body weight daily per os

The polycythaemic effect of cobalt can be prevented or reduced by many substances Ascorbic acid, EDTA, choline, cysteine, histidine, methionine, methylen blue and nicotinamide have such properties and therefore must be taken into account when cobalt is administered These substances have been listed earlier (7, 8)

The effect of cobalt is thus highly dependent on the amount administered and its chemical combination Little is known about the mode of action of cobalt and the amount needed to obtain an optimal result This experiment was designed to reveal the minimal amount of cobalt capable of producing polycythaemia in the rat

MATERIAL AND METHODS

25 adult male albino rats of the Wistar strain were used The animals weighing from 204 to 250 grams were divided into 5 groups of 5 rats each The mean weight of the animals was 230 g

Cobalt cloride was dissolved in 0 9 per cent saline and injected subcutaneously Rats in group I were treated with 0 4 mg of cobalt per kilogram of body weight daily, 6 days a week group II with 0 2 mg/kg group III with 0 1 mg/kg and group IV with 0 05 mg/kg Rats in group V served as a control and were injected with 1 ml of saline daily The treatment was continued over a period of 5 weeks Blood samples were obtained from tail veins and the haemoglobin determination and erythrocyte count were made before and after 5 weeks of cobalt treatment Subsequently the animals received no treatment for 3 weeks and determinations were repeated in order to study the disappearance of the polycythaemic reaction The weight of the animals was checked throughout the experiment

RESULTS

The results are summarized in Figure 1, which shows the changes in the mean haemoglobin and erythrocyte count in each group

After 5 weeks of cobal administration a significant increase in haemoglobin and erythrocyte count is seen in groups I, II, and III, treated with *0 4, 0 2 and 0 1 mg of cobalt per kilogram of body weight* daily In group I, the increase in haemoglobin is 39 per cent and the erithrocyte count 46 per cent In group II the corresponding values are 37 per cent and 53 per cent and in group III 24 per cent and 28 per cent In group IV the haemoglobin remained unchanged but an *increase of 13* per cent in seen in the erythrocyte count In the control group an in-

FROM THE CHICAGO MEDICAL SCHOOL DIVISION OF ONCOLOGY
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HAIR SUCCESSION AND ITS INTERFERENCE WITH PAPILLOMA FORMATION DURING TUMOR PROMOTION IN MICE

By

KAI DAMMERT[1]

Received 9 xii 61

Berenblum et al in 1958 reported some experiments analysing the significance of hair cycle phase in relation to the stages of initiation and promotion of experimental carcinogenesis in mice They arrived at the conclusion that both stages remained essentially unaffected by the phase of the cycle Their results with one application of the carcinogen confirmed, however, the earlier findings of *Andreasen & Engelbrecht-Holm* 1953, *Borum* 1954 (5), *Klinken Rasmussen* 1955, *Sherwin-Weidenreich, Herrman & Rothstein* 1959, according to which applications made in the resting phase of the cycle yielded more tumors than those made in the growth phase This "follicle cycle effect" was explained by *Berenblum et al* as an inhibition of the promoting action of the carcinogen as a result of its short retention in the skin at the growth phase as shown by fluorescense microscopy The responsiveness of the skin to the chemical was thought to be the same in both resting and growth phases The object of this paper is to report some cyclic effect during promotion with daily applications of a potent chemical promot[illegible] of hair [illegible] the gro[illegible] during the succession of the hair generations and making it easy to follow the biology of the tumors in relationship to the hair growth waves As is known the tumors in such an experiment are mostly papillomas of short lifespans involving fluctuations of sizes and shapes Consequently they are well suited to indicate every kind of alterations in their growth conditions

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haemoglobin beyond the 20 per cent obtained with 1 25 mg/kg in 4 weeks Furthermore, when the daily intake of cobalt exceeds 0 2 mg/kg the erythropoietic effect seems to be reduced This phenomenon is due obviously to the toxicity of cobalt Many toxic symptoms, including weight loss, observable already when the dose of cobalt exceeds 1 25 mg/kg per day (8, 10) This general toxicity may interfere with the erythropoiesis and prevent the attainment of the maximal effect This assumption is in no way incompatible with the theory of the erythropoietic effect of cobalt being the result of an inhibition of some enzymes and a consequent tissue anoxia, both of which, of course, are to be regarded as toxic manifestations The only important point is whether other systems besides the haematopoiesis are involved because these are highly dependent on the quantity of cobalt administered

SUMMARY

Cobalt chloride ($CoCl_2$ $6H_2O$) dissolved in 0 9 per cent saline was administered subcutaneously to adult male albino rats daily over a period of 5 weeks Even a dose of 0 05 mg of cobalt per kilogram of body weight daily had a slight polycythaemic effect 0 2 mg of cobalt per kilogram of body weight produced an optimal erythropoietic effect By increasing the dose of cobalt above this amount no additional erythropoietic response is obtained

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Tween 60 was applied undiluted at 36° C with a regular glass dropper One drop was spread evenly over the experimental field

The mice were examined twice a week The growth areas were recognized by keeping the experimental field always shorn using electric clippers and or scissors When the growth wave passed the hair had to be removed at every examination At resting phase this procedure was not needed The delay in recording the commencement of the growth phase was compensated by a corresponding delay in

TABLF 1

The Average Duration of the Hair Growth and Resting Periods of the Successive Hair Generations in the Centre of the Experimental Field and the Total Number and Regressions of the Tumors Recorded on the Whole Experimental Field during the Stage of Tumor Promotion

	Number studied generations	Average duration of the periods*		Tumors	
		Resting	Growth	Total Number	Number of regressions
Normals animals two months old at the beginning of the experiment	2	52 0	8 2		
	2	57 5	10 0		
	2	54 0	10 0		
	2	50 5	10 0		
	2	43 5	8 5		
After initiation with 20 µg DMBA in acetone	2	49 0	10 0		
	2	59 0	10 0		
	2	57 5	8 5		
	3	39.3	9 0	1	–
	3	34 7	10 0		
During daily applications of Tween 60 beginning one week after iniation with 20 µg DMBA in acetone	7	24 1	10 1	2	1
	6	20 2	10 0	1	–
	11	12 5	10 2	3	2
	5	19 4	8 2	–	–
	11	12 2	9 2	6	5
	6	19 2	12 0	1	–
	10	16 4	10 1	8	5
	10	16 2	8 5	–	–
	11	14 0	10 5	23	14
	11	13 7	8 9		–
	9	17 8	10 2	–	–
	8	20 8	9 6	–	–
	8	21 8	10 6	1	1
	1	10 0	10 0	–	–
	8	13 3	10 8		–
	6	12 7	9 5		
	10	11 9	10 4	5	5
	8	14 5	9 6	10	5
	9	12 9	10 4	1	–
	5	13 8	11 6	–	–
	9	19 2	8 7	3	2
	8	16 0	10 1	9	3
	9	17 7	9 8	7	1
	4	29 5	13 0	6	o
	7	29 9	9 7	1	
	10	19 8	7 0	1	1
	9	18 3	10 0	1	1
	9	16 9	9.3	4	3
	8	14 4	11 5	4	4
	5	27 0	12 4	9	7

* Days

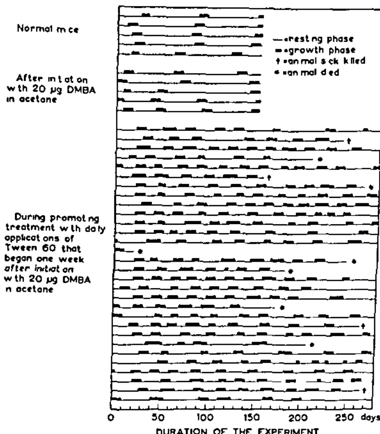


Fig 1

In all groups was the growth phase approximately equal in duration through the *time of observation During the promotion stage of tumor production the resting* phase was markedly curtailed and the hair succession thus speeded up

MATERIALS AND METHODS

Female Swiss albino mice bred at random in this laboratory were used The animals were two months old and weighed 22 25 g at the beginning of the experiments They were kept in plastic cages on wood shavings and fed Rockland diet in pellets and water ad libitum An area of 1 5×1 5 cm on the upper back was shaved with an electric clipper one week before application of the chemicals In order to avoid dislocations of the treated field on the back and to facilitate recognition of the individual tumors in this field small dots of india ink was tattoed in the midline on the upper and lower border of the experimental field The dots were studied histologically in some of the animals at the time when treatments were instituted For this purpose the animals were killed and the total of the skin on the back was divided at the midline and embedded in paraffin In sections the india ink was found located mainly immediately underneath the dermis No signs of reactive changes were seen locally

The chemicals used were 9 0 dimethyl 1 2 benzanthracene (DMBA) (Eastman Organic Chemicals) purified by chromatography and polyoxyethylene sorbitan monostearate (Tween 60) (Atlas Powder Co) DMBA was dissolved in acetone of reagent grade distilled before use 20 µg was applied with a calibrated micropipette

As shown in Table 1 the tumor yield was essentially ascribable to the multiplicity of tumors in approximately one third of the animals Here also most of the regressions occurred It may be noted from Table 2 that during the first 20 weeks a clear preponderance of tumor appearance in the resting phase and disappearance in the growth phase was discernible Later this trend was no longer obvious In some of the animals presenting a crop of tumors early in the period of promoting treatment this phenomenon was very clear The tumors developed almost simultaneously in a resting period and grew rapidly until a wave of hair growth reached them A regression would set in then and many would disappeare before growth phase around the tumors had finished As shown in Table 1 this effect was not of an order admitting of the average duration of resting and growth periods to reflect any difference in the final total amount of tumors in the individual animals

DISCUSSION

Dry (1926) and *Borum* (1954 a) outlined the spread of the different generations in the pelage of normal mice Little attention was paid to the duration of the cyclic phases of participant individual hair follicles *Dry* observed many one month cycles *Glucksmann* (13) and *Wolbach* (22) estimated the duration of the growth phase to 21 days Approximately the same figure is given by Montagna (pp 236 and 239 240) *Wolbach* (op c) found the resting period between growth waves to be very short *Andreasen* (1) stated that although individual variations were great the growth phases lasted approximately for 10 days the resting phases for longer periods The last mentioned opinion on normal pelage has been substantiated in the present study The growth phases covered about 10 days the resting intervals usually being 5 times longer However the early anagen lasting according to *Chase et al* (10) for 5 6 days must be taken into account during which period hair tips are not visible on the surface of the skin This will lenghten the actual growth period up to 15 16 days and shorten the intervals by the corresponding time The application to the skin of an initiating dose of DMBA did not disturb this ordinary rhythm

With the institution of the promoting treatment a much faster change of the coat commenced The duration of growth phases remained unaltered but the duration of resting periods was reduced to a fraction of the normal In some animals the length of resting periods tended to equal the time for growth Growth of the hair follicles seemed to be easily stimulated by the chemical This pattern of behavior is well known subsequent to hair plucking and to local damage of different origins (4 9 11) Besides the absence of any substantial change in the usual duration of growth periods strongly indicates that the chemical did not damage the important functional parts of the follicles to such a degree as to interfere with the normal hair forming ability

recording the cessation The growth areas were recorded, together with tumors on special diagrams and recharted at every examination taking into consideration any changes of the growth areas and of the number, size, type and position of the tumors As indicator of the phase influence on the tumors the rate of appearance of the papillomas during resting and growth phases appeared to be usable

Only the first hair generations were spreading evenly and rather simultaneously over the treated area Later the growth wave became disorderly and more slow eventually becoming patchy An appraisal of the involvement of individual follicles as participants in the succeeding pelages could therefore be achieved only by choosing a limited, constant spot as an area of observation The situation in ½ square centimetre centrally in the experimental field was marked on the diagrams at every checking Only whole generations including both resting and growth phases in pairs were included in the final records In this way the preterminal period of illness characterized by marked retardation or dessation of the cyclic activity, could be eliminated from the results

For the study of the cyclic changes in the normal pelage a group of 5 animals was used The effect of initiation was studied in an other group of 5 animals The growth pattern in both groups was followed for two or three successive hair generations In the main series 30 animals were used and the hair growth together with appearance and disappearance of the tumors were followed during 40 weeks of promoting treatment

RESULTS

1 Succession of Hair Generations See Fig 1 and Table 1

a) *Normal animals* Table 1 presents data related to two pelages (G III – G IV) completely followed This required periods of at least 20 weeks The duration of the resting periods is seen usually to cover 7 8 weeks and durations of growth periods about 10 days Considerable, individual variations were noted as regards the duration of the resting periods

b) *Initiated animals* No significant difference was seen as compared with the normal animals

c) *Initiated animals during tumor promotion* The promoting treatment curtailed the resting periods in all of the animals, bud did not influence the duration of the growth periods The hair generations followed each other at short intervals In some animals the resting periods occasionally were even shorter than the growth periods

2 The tumors and their dependence on the cyclic phase

TABLE 2

The Distribution of Appearance and Disappearance of Tumors in the Experimental Field during the Resting and Growth Phases during Promotion in the Actual Group of 30 Mice

Appearance				Disappearance			
Resting phase		Growth phase		Resting phase		Growth phase	
0 20 weeks	20 40 weeks	0 20 weeks	20 40 weeks	0 20 weeks	20 40 weeks	0 20 weeks	20 40 weeks
51	22	14	16	9	16	23	17

was accomplished by a single application of 20 μg of 9 10 dimethyl-1 2-benzanthracene (DMBA) in acetone and promotion was accomplished with daily applications of polyoxyethylene sorbitan monostearate (Tween 60) The method employed included careful clipping and examination twice a week

2) Normally the resting periods estimated by this method lasted for about 50 days and the growth phases for 10 days The initiation did not change the normal timing of resting and growth phases

3) The promotion caused a speed up of the cycle by a marked abbreviation of the resting periods

4) During the first 20 weeks of promotion the tumors seemed to show regressive features *e g* a reduction of size during the growth phase Furthermore most of these appeared during the resting phases to disappear during the growth phases

5) The results are discussed

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Resting phases during the first 20 weeks of promoting treatment favoured the development of papillomas During prolonged tar paintings and repeated applications of carcinogenic hydrocarbons, the appearance of tumors as related to the cyclic phases has been described Most of the reports provide vague evidence of a positive correlation between growth phases and tumor development (14, 15, 21) *Borum* (6) found trends of the same correlation when tumors were produced by one application of DMBA Only one report records a diminuation of the size of epidermal tumors when the surrounding skin amount entered the growth phase (*Mottram* 1945)

The question now arises why the results are different The discussion on the follicle cycle effect on tumor development must always consider two factors 1) differences, if any, of susceptibility and responsiveness of the skin and 2) differences, if any, of peneration and retention of a tumorigenic chemical during the two stages of the hair cycle The latter also includes that theoretically, dilution of the carcinogen may be different in the individual phases as discussed by *Klinken-Rasmussen* 1956 (pp 65-69) Indeed, many of the reports have taken into consideration the biological phase differences According to *Mottram* (op c) the number of mitotic metaphases in the epidermis was greatly reduced during the growth period caused by the active hyperaemia around the growing follicle bulbs and the corresponding anaemia in the upper part of the skin Many other histological and histochemical epithelial and dermal differences during the two phases have been reported (*e g* 7, 8, 10, 17) The increase of the subcutaneous fat tissue in the growth phase is generally known Although some of these data are contradicted by the results found by other workers (11, 16), it can hardly be denied that many of the life processes in the skin are different in the resting and the growth phases This aspect is actually the only background for phasic interference in cases of tumors produced by one application of a chemical carcinogen as in the experiment of *Borum* 1958 *Mottram s* experiment (1945), in which converse results were obtained, can not be assessed, because it is not recorded whether the benzpyrene had been used once or repeatedly

By promotion with a chemical used daily the situation might be entirely different from the conditions mentioned above Different penetration and retention of the chemical in resting and in growth phases must then seriously be accounted for Evidence to that effect is already available (11) Therefore it is obvious, that the rôle played by the phases of the hair cycle must be different when different methods of tumor production are used

SUMMARY

1) The effect of initiation and promotion on the periodical hair growth together with the reciprocal influence of the cyclic phases on tumors in the skin of the back of mice have been studied Initiation

was accomplished by a single application of 20 μg of 9 10 dimethyl 1 2 benzanthracene (DMBA) in acetone and promotion was accomplished with daily applications of polyoxyethylene sorbitan monostearate (Tween 60) The method employed included careful clipping and examination twice a week

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FROM THE CHICAGO MEDICAL SCHOOL DIVISION OF ONCOLOGY, CHICAGO, ILLINOIS

THE MORPHOLOGY OF THE HAIR FOLLICLES DURING TUMOR PROMOTION IN MICE

By

KAI DAMMERT[1]

Received 9 xii 61

Many students of experimental carcinogenesis (1, 6, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22) have considered hair follicles to be intimately involved in the tumour formation of the skin The discovery of a follicle cycle in the coat of experimental animals which continue to function also in the treated area far beyond the time of appearance of tumours and the demonstration that the phase of the cyclic activity in some way interfered with the tumour formation, bring up new questions considering the mechanism of this interference In the preceding paper (7) it was shown, that the conditional papillomas during promotion in a two stage experiment tended to grow during the resting phase and to diminish and even disappear during the growth phase of the hair growth cycle A priori this behaviour of the papillomas seemed to be caused by the increased vulnerability and responsiveness of the skin to chemical influences during the resting phase as shown before by many workers (2, 4, 17) The question in relation to tumour promotion is studied morphologically in this report

MATERIALS AND METHODS

Female Swiss albino mice bred at random in this laborator[illegible] animals w[illegible] ment The [illegible]

(Tween 60 [illegible]

[illegible] of DMBA was applied with a micropipette Tween 60 was applied with a standard glass dropper, each drop measuring approximately 20 µl beginning one week after the initiation Two drops

This investigation was [illegible] Sinai, Snell

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Fig 1

Anagen phase as seen in an initiated animal 2 days after the start of the promotion with daily applications of Tween 60 Growth wave spreading in the cranial direction i e towards the right in the microphotogram The growing follicles spaced with high interfollicular domes are well exposed in the caudal field to the left The surface is continually more even towards the right where the growth phase just has commenced H & E × 25



Fig 2

The mode of loss of a hair in the resting phase during the stage of promotion one week after commencement of daily application of Tween 60 The club hair and its capsule are pushed up by the swelling and proliferation of the surrounding cells of the epithelial sac Snook × 210

were spread to cover the whole experimental area Two or three of the animals were killed each time at intervals of ½ 1 2 4 10 and 20 weeks after the institution of treatment with Tween 60 When sacrified the animals had been treated for the last time the day before death

A second group of 15 animals received the same preparatory treatment with 20 µg DMBA followed one week later by Tween 60 two drops being applied twice weekly Two or three animals were killed each time at intervals of ½ 1 2 4 and 10 weeks after the commencement of the secondary treatment When tthe animals were killed the last treatment had been made 3 days before

The animals were carefully autopsied and the different organs histologically studied If changes suggesting illness were found the animal was excluded from the final counts At autopsy the skin was fixed in Susa fixative for five hours and subsequently divided along the midline, both halves were embedded in paraffin The sections 5 µ thick were stained with haematoxylin and eosin PAS and reticulum stain according to Snook

Terminology The anatomy of human hair growth has been extensively studied since the middle of the last century Many authors have inoculated their own terms and hence some confusion has ensued which has been disturbing when the terms have been applied also to the follicles of experimental animals According to *Dry* (9) the hair cycle of the mouse consists of a telogen an anagen and a catagen phase

The resting follicle consists of a rather even sleeve of epithelial cells It is slanted downwards and forward In the middle of the hind wall or a little higher up it is joined by the duct of the sebaceous gland Between this point and the epidermis the neck of the follicle is seen A bulb is lacking, instead the bottom of the epithelial tube contains the club of the hair enclosed in a sac of epithelial cells i e the epithelial sac The cells adjacent to the club are partially keratinized and are called the capsule The club in anchored between the cells of the capsule with delicate keratinized fibrous processes Sometimes a collection of cells are seen in connection with the sac on the side of the follicle forming the obtuse angle to the surface It is identical with the bulge and serves as insertion of the arrector muscle An attenuated cord of cells below the epithelial sac is called the hair germ Immediately underneath it is the dermal papilla The deepest extension lies in the lower part of the dermis

A bulb of the anagen follicle is located deep in the subcutaneous fat An invagination at the base contains the papilla The hair is growing from this bulb together ... an
and closely connected with its inner
epithelial tube called the outer root
outer root sheath fuses with the s
swelling called the bulge to which the arrector muscle is attached the bulge usually containing the epithelial sac of the club hair of the former hair generation The narrow area between the bulge and the duct of the sebaceous gland is called the isthmus It is lined by a peculiar hyaline derivative of the inner root sheath The outer root sheath borders with a hyaline membrane the glassy or vitreous membrane towards a connective tissue sheath the outermost structure of the follicle

The catagen phase involves a sudden tearing down of the active part of the anagen ... formation and
follicle l ... he papilla cells
the rele ... epithelial sac
are seer
around

RESULTS

No qualitative differences between the two experimental groups were found Consequently they shall be considered together

Telogen phase During this phase the skin generally was desquamating rough and not infrequently eroded During the first resting periods extensive depilation often occurred Generally the walls of the resting follicles were thicker than the corresponding parts of the active follicle The sebaceous glands were round and consequently their lowermost extensions were located higher up in the dermis than had been the


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case during the growth phase This made the interfollicular epidermis look quiescent (Fig 1) In areas of epilation, the loss of hair was caused by proliferation of the cells of the epithelial sac, which consequently *pushed up the hair with its capsular cells (Fig 2)*

Anagen phase: At this phase the interfollicular epidermis formed high domes between the follicular mouths The follicles were long rigid tubules A remarkable traction downwards was mediated by the connective tissue sheath to the site of the sebaceous gland and the bulge with the insertion of the muscle Consequently the gland was elongated and compressed in the narrow angle formed by the wall of the isthmus, the bulge and the arrector muscle (Fig 3) Supported by an extension of the glassy membrane and the connective tissue sheath and filled with the growing, new hair and usually also with the club hair of the former hair generation, the space of the isthmus was tightly packed The impression of a regular locking effect in this very important region was completed by the corrugated PAS positive inner lining of the isthmus (Figs 3 and 4) After approximately 10 weeks of treatment the sebaceous glands very often showed own secretory ducts directly to the surface

In the transient part of the follicle below the sebaceous gland never trends of alterations and reactive changes were observed From the upper part of the bulb, the slender inner sheath and larger outer root sheath followed in a regular way the hair root upwards In spite of the daily treatment with large quantities of the strong promoter substance Tween 60 the bulb was allways well preserved (Fig 5) A considerable dilatation and congestion of the blood vessels in the adjacent connective and fat tissue represented a constant finding

Catagen phase The club hair formation always began rapidly Only rarely was it possible to follow the events of this phase A quick separa

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Fig 3-6

Fig 3 Anagen phase 3 days after the first promoting treatment with Tween 60 In the region of the isthmus the sebaceous gland is pressed aginst the hair canal in which a growing hair emerges Note the close connection of the corrugated inner lining of the isthmus and the hair In the lower border under the bottom of the sebaceous gland the bulge is seen PAS × 210

Fig 4 Anagen phase after 20 weeks of promotion with daily applications of Tween 60 The narrow isthmus is seen to be supported by the argyrophilic vitreous membrane which *from the intumescense of the bulge is tapering upwards* encircling the outer root sheath in the fissure between the sebaceous gland and the wall of the follicle Snook × 210

Fig 5 The bulbus in an anagen phase after 2 weeks of daily promoting treatments with Tween 60 No signs of disturbances in the normal cellular structures H & E × 210

Fig 6 Catagen phase after 20 weeks of promotion with daily applications of Tween 60 The dissociation of the papilla and the club hair capsule is shown The outer root sheath cells are encircled by a thick and wrinkled glassy (vitreous) membrane A thickened part of the connective tissue sheath is seen around the diminishing bulb PAS × 210


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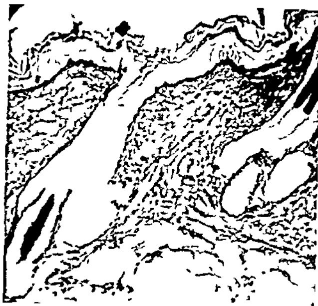


Fig 7
Normal follicular structures at the end of the catagen phase after 20 weeks of promotion with daily application of Tween 60 Snook × 210

tion between the club hair formation and the dermal papilla cells was accompanied by atrophy of hair matrix cells and release of the dermal papilla, quite in the same way as is seen in the normal follicle (Fig 6) The simultaneous thickening of the glassy membrane and of the connective tissue sheath was conspicuous The impression of axial tension along the tube ceased continually when the club of the hair and the root sheaths were emerging upwards Also the bottoms of the sebaceous glands raised and finally the lobes of the glands resumed their round and quiescent appearance as earlier in the resting phase Thus repeatedly after 20 weeks of daily treatments with Tween 60 catagen phase finished with a very orderly appearance of the follicles (Fig 7)

The phase patterns and the tumours In the group of daily treatments with Tween 60 papillomas developed at the site of treatment in some of the animals before they were killed In the sections they were seen to be formed by fibroepithelial hyperplasia of the upper parts of the skin, which was lying above the level of the sebaceous glands Thus the glands seemed to be located even deeper than usually seen during the growth phase and always underneath the active tumorigenic process If the skin area to contract the tumor were in the active growth phase at the time of study, also the follicles in the tumor would be in a growth phase In other words the hair cycle continued in the papillomas during the time of sacrifice (Fig 8)



Fig 8

A fibroepithelial papilloma after 10 weeks of promotion with daily applications of Tween 60 The tumour is composed of slender exophytic papillary projections covered by acanthotic homologues of former follicular walls corresponding to the permanent parts of the follicles Consequently sebaceous glands are seen under the base of the tumor A dilated capillary vessel appears in the lower border between two anagen follicles H & E × 100

DISCUSSION

From the study emerges the impression already pointed out by *Chase & Montagna* (4), that a growth phase must be considered coexistently as a creative state and as protective arrangement of the skin Certainly the upper hair canal, which during the growth phase contains 2 hairs and much sebum, squeezed mechanically from the compressed glands, present more difficulties for the penetration and retension of the chemical agent, than does looser structure in the resting phase A decrease of the sebaceous glands during the growth phase has been observed also by *Chase et al* (5) The isthmus repre-

sents a very effective barrier which precludes the chemical agent from access further down the root sheaths. With regard to the morphological circumstances in the transient part of the follicle, this would be difficult any way, because there is a close connection or direct cohesion between the hair root and the sheaths. In this study it has not been possible in the growth phase to show any stalled hairs or squamous metaplasia and keratinisation of the outer sheath, as has been observed by *Wolbach* (22). But this author used 0 3 and 0.15 per cents solutions of benzpyrene in benzene repeatedly, and it is hardly possible to compare results obtained with these different methods. Certainly the toxic effect of the chemical agent can be exaggerated to such a degree, that even in the growth phase the region of the isthmus and the bulge including the important hair germ cells are to be destroyed, and the follicle to be converted in to a permanently open, tubular or cystic, structure Naturally such degree of damage prevents any future follicular cycle effect

Another factor deserving consideration in the follicular effect during tumor promotion is the subcutaneous hyperaemia seen during the growth phase. It is possible, as suggested by *Mottram* (16), that the follicle bulbs are fully provided with blood, only during the growth phase thus leaving the surface, containing the papillomas, in a state of relative anaemia with consequent regression of the papillomas

Finally it is interesting to recall that in contrast to the effect of chemicals, moderate doses of x-rays are known to injure the hair matrix and to stop hair growth in the growt phase, but also to leave the resting follicle unaneffected (3, 4) This effect may simply depend on the selective vulnerability of the young, proliferating hair matrix cells due to x-rays in accordance with the effect of these rays on comparable tissues in other parts of the body.

SUMMARY

1) The hair follicles have been studied histologically during the stage of tumour promotion The experimental area on the upper back of mice was initiated with 20 μg of 9 10-dimethyl-1 2-benzanthrazene (DMBA) After one week the promoting treatment with polyoxyethylene sorbitan monostearate (Tween 60) was started One group received the secondary treatment daily, another group received it twice weekly In the former group 2 or 3 of the animals were killed each time at intervals of ½, 1, 2, 4, 10 and 20 weeks, in the later group at intervals of ½, 1, 2, 4 and 10 weeks The treated skin was divided along the midline and embedded in paraffin Sections were made from both sides longitudinally The changes in both groups were qualitatively uniform, although they were more marked in the former than in the latter group

2) Resting follicles were especially vulnerable during the first week

The walls showed considerable acanthosis Occasionally the prolifera tion was strong enough to cause a loss of the club hair and its capsule Later the acanthosis of the follicular walls still remained more marked in the resting phase than in the growth phase suggesting better penetration of the chemical promoter and explaining the better tumour growth in the resting phase

3) In the anagen phase the follicles were long and rigid as they are normally The sebaceous glands had been retracted downwards, seemingly by a tension of the connective tissue sheaths, and consequently were compressed against the narrow isthmus of the follicle, which because of special structures was practically impermeable for chemicals The funnel of the neck of the follicle was crowded by the hair and the sebum from the sebaceous glands No change caused by the chemicals could be seen in the deeper transitory part of the follicles The lack of penetration into and retention in the follicular structures of the acting chemical promoter is considered the reason for the re gression of tumors in the growth phase shown in the preceding paper

4) The papillomas were formed at supraglandular level and their regression in the growth phase was supported obviously also by the distribution of the blood mainly to the subcutaneous tissue during this phase

5) The follicular structures of the catagen phase showed normal patterns throughout the experiment

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GROWTH AND SPREAD OF ROUS RAT SARCOMA

By

TOM SALDEEN

Received 29 xii 61

Recent investigations (*Ahlström & Jonsson* 1962) have shown that inoculation of newborn rats with Rous chicken sarcoma (Schmidt-Ruppin strain) is very often followed by the development of a sarcoma at the site of injection, that the rat sarcoma probably is induced by a variant of Rous sarcoma virus; that the rat sarcoma can be transplanted serially in rats but that, like other transplantable rat tumours it can only be transferred to new rats by cellular material

This paper is concerned with an investigation of the growth and spread of the transplanted Rous rat sarcoma (R R sarcoma) and a comparison between this tumour and a well-known transplantable rat tumour, Walker carcinosarcoma

MATERIAL AND METHODS

Animals —Female [illegible]

[illegible] ss steel screen and 0 I U of penicillin, [illegible] was kindly supplied by prof [illegible]

[illegible] a short [illegible] loop was pulled out through the incision and the tumour suspension was injected into the mesenterial *radix* with a 22 gauge needle The injection was performed under a Zeiss binocular dissecting microscope

Intraportal injection —The rats were anaesthetized with ether [illegible] abdominal cavity was opened [illegible] out through [illegible] was pulled [illegible] mesenterial [illegible] into a large [illegible] blood into [illegible] nt escape of [illegible] is carefully compressed for about 2 [illegible] The injection was performed under [illegible]

[illegible] veins was performed by means of a 14 gauge needle

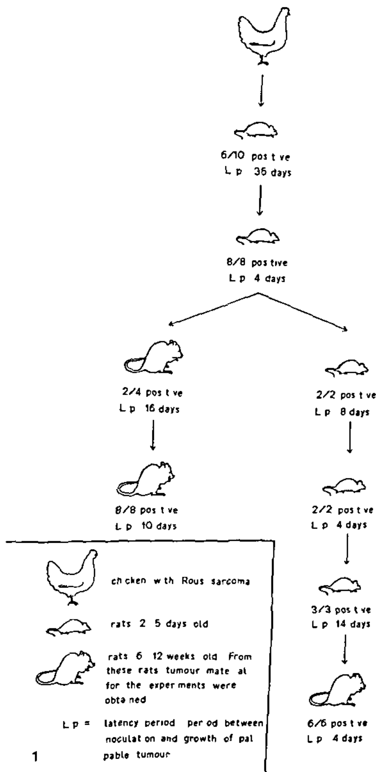


Fig 1
Transplantati n scheme c f R R sarcoma

Measurement of tumour size—The size of the intramuscular subcutaneous and intramesenterial tumours was assessed on the basis of the lengths of the 2 longest perpendicular diameters of the tumours

Determination of the number of tumour cells in the peritoneal fluid—The tumour cells in ascitic fluid diluted 1 100 with physiological saline solution containing 0 05 per cent eosin were counted in a Buerker haemocytometer

Histopathological examination The lungs liver kidneys spleen lymph nodes (axillary mediastinal upper and lower para aortic) were routinely examined In the animals inoculated intraperitoneally, the greater omentum mesentery, peritoneum diaphragm parasternal tissue and fat tissue around the tail of the pancreas and coecum were also examined Any other organs whose gross appearance suggested the possibility of tumour infiltration were also studied The sections were stained using haematoxylin and eosin

RESULTS

Intramuscular Transplantation

Seven adult rats (weighing about 200 g) and 7 young rats (weighing about 50 g) were injected intramuscularly in the right thigh with 1 ml and 0 25 ml, respectively, of a suspension of R R sarcoma from the 2nd rat passage

TABLE 1

Frequency of Takes Deaths and Metastases in Animals Injected with R R Sarcoma by Various Routes

Rats	200 g	50 g	200 g	200 g	200 g	50 g	200 g	200 g
Mode of transplantation	intra muscular	intra muscular	subcutaneous	intra mesenterial	intra peritoneal	intra peritoneal	intra portal	intra venous
Tumour dose	1 ml	0 25 ml	1 ml	0 1 ml	1 ml	1 ml	0 1 ml	0 1 ml
Positive rats	6/7	7/7	2/7	6/7	13/16	5/8	7/7	6/7
Spontaneous death	0/6	5/7	0/2	6/6	13/13	5/5	7/7	5 6
Growth in para aortic lymph glands	1/6	1/7	0/2	1/6	7/13	1/5	0/7	0 6
Growth in mediastinal lymph glands	0 6	0/7	0/2	2/6	8 13	2 5	0/7	1 6
Growth in liver	0/6	0/7	0/2	4 6	6/13	1/5	7/7	0 6
Growth in lungs	0 6	3/7	0/2	4 6	3/13	1/5	0/7	6 6

In 6 of the adult rats tumours developed at the site of injection and assumed a mean size of 15 mm × 15 mm within 10 days (Table 1) On the 20th day the tumours were on the average 25 mm × 30 mm By then however, most of these had commenced to regress and on the 30th day, when the rats were killed, the mean size of the tumours was 25 mm × 25 mm No rat died before the 30th day The tumours were roundish grayish red, fairly soft They were not encapsulated, and the cut surface often showed large central areas of necrosis Histologically, they were built up of densely crowded, elongated cells with an elongated chromatin rich or, fairly roundish vesicular nucleus with one or several nucleoli (Fig 2) The tumour infiltrated the skeletal muscles

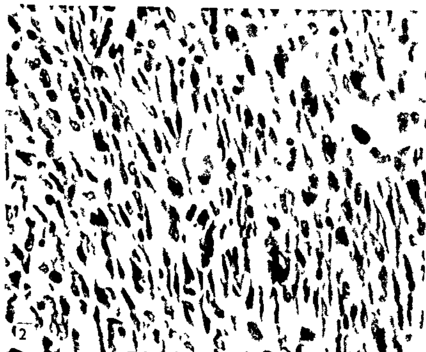


Fig 2

Growth of R R sarcoma following intramuscular injection Part of tumour dominated by spindle shaped cells Htx eosin × 480

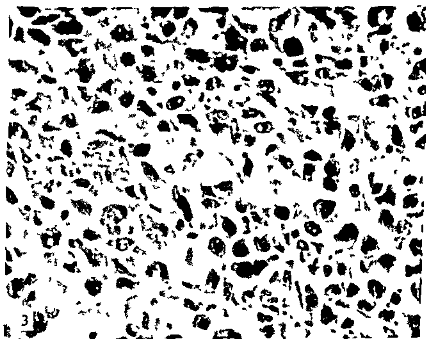


Fig 3

Growth of R R sarcoma following intramuscular injection Part of tumour dominated by polymorphous cells Htx eosin × 480

In other areas the tumour was polymorphus with roundish or polygonal cells with a wide variety of nuclei (Fig 3)

The regional lymph nodes were often considerably enlarged but microscopically they generally showed only nonspecific lymphadenitis Only one rat showed metastases in the regional lymph nodes No secondary growths were found in any of the other organs

All of the young rats developed tumours at the site of injection (Table 1) The tumours grew rapidly and progressively and 5 of the rats died between the 18th and 30th day after the inoculation The remaining two young rats were killed on the 30th day The mean size of the tumours was 25 mm × 20 mm In contrast to the tumours in the adult rats necrosis was rarely seen Histological examination showed the picture of a spindle cell sarcoma

One rat showed metastases in the regional lymph nodes Three rats had widespread secondary growths in the lungs The metastases showed the same histological structure as the primary tumours No secondary growths were seen in any of the other organs studied

Subcutaneous Transplantation

Seven adult rats (weighing about 200 g) were injected subcutaneously with 1 ml of the same tumour suspension as the one used in the intramuscular experiments Within 10 days all of the rats showed tumours at the site of injection The growths were on the average 10 mm × 10 mm They grew slowly and 14 days after the injection most of them had started to regress On the 30th day when the rats were killed only 2 showed tumours which were 20 mm × 30 mm and 3 mm × 3 mm No rat died before the 30th day The tumours were roundish encapsulated and their cut surface showed widespread central cystic necrosis Histologically only a small peripheral zone of vital tumour tissue was seen The tumour had the same histological structure as the tumours in the adult rats which had been injected intramuscularly No metastases were seen in any of the organs studied

Intramesenterial Transplantation

Seven adult rats (weighing about 200 g) were injected with 0 1 ml of a suspension of R R sarcoma into the mesenter [illegible]

[illegible] occupy [illegible] major part of the abdominal cavity In one of the rats injected with a suspension from the 3rd tumour passage the tumour did not take The cut surface of the tumours was grayish white and showed a central necrosis The histological picture was dominated by spindle cells but in a few areas the sarcoma was more polymorphocellular

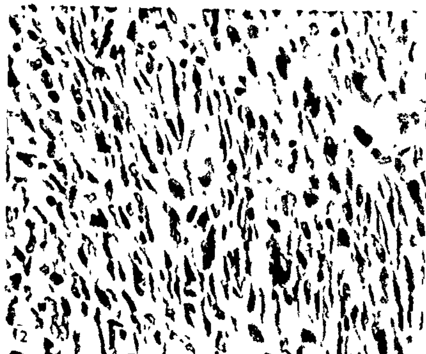


Fig 2

Growth of R R sarcoma following intramuscular injection Part of tumour dominated by spindle shaped cells Htx-eosin × 480

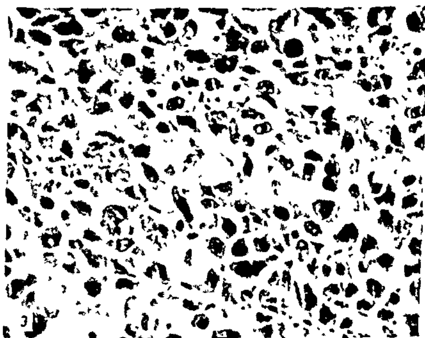


Fig 3

Growth of R R sarcoma following intramuscular injection Part of tumour dominated by polymorphous cells Htx eosin × 480



Fig 5

Rat with R R sarcoma following intraperitoneal injection Observe extensive infiltration of greater omentum

wall of the abdominal cavity, and never the small pelvis The tumour had thus spread mainly to the upper part of the abdominal cavity (Fig 7) The fatty tissue around the tail of the pancreas was markedly invaded sometimes also the pancreatic parenchyma, and less frequently the fat around the coecal pole More than half of the rats showed metastases in the upper para aortic lymph nodes (renal nodes) No metastases were found in the lower para aortic lymph nodes (lumbar nodes) Sometimes the tumour had invaded the surface of the liver, occasionally it had grown into the liver hilum and sometimes into the *vena portae* (Table 1) Sometimes it had invaded the hilum of the spleen but it had never grown through the vessels there As a rule, the tumours spread outside the abdominal cavity, and often invaded the parasternal tissue, particularly the lymph vessels (Fig 8) In 8 of the rats the tumour had spread to the anterior mediastinal lymph nodes The lungs showed no gross metastases but histological examination revealed intravascular deposits in 3 of the rats Some of the extraperitoneal secondary growths were spindle shaped while others were of the polymorphocellular type of sarcoma

Five of the young rats died between the 10th and 13th day after injection at which time they showed 20 30 ml of ascites of the same



Fig 4
Smear of ascitic fluid from rats with growth of R R sarcoma following intraperitoneal injection Papanicolaou × 2100

Four rats had metastases in the liver and 3 had peritoneal carcinomatosis Four of the rats showed widespread pulmonary metastases The secondary growths were of the same histological character as the primary tumours

Intraperitoneal Transplantation

Sixteen adult rats (weighing about 200 g) and 8 young rats (weighing about 50 g) were injected intraperitoneally with 1 ml of a tumour suspension from the 5th rat passage

Thirteen of the adult rats died between the 12th and 16th day after the injection (Table 1) The abdominal cavity contained 40–100 ml of blood-stained fairly watery fluid, containing 4–10 × 10⁶ tumour cells per ml (Fig 4) The number of inflammatory cells varied between 25 per cent and 150 per cent of the number of tumour cells Some 10–25 per cent of the cancer cells were stained with eosin, *i e*, they were dead The entire greater omentum was infiltrated with tumour (Fig 5) The diaphragmatic musculature, particularly on the right side was also infiltrated (Fig 6) Tumour nodules were often seen in the mesenterium of the small intestine In none of the animals had the tumours spread to the lymphatics or lymph nodes in the mesenterial *radix* The tumour had sometimes invaded part of the peritoneum lining the upper parts of the lateral walls of the abdominal cavity, less frequently the caudal parts of the lateral walls of the abdominal cavity, rarely the ventral



Fig 8
Parasternal lymph vessel from a rat following intraperitoneal injection of R R sarcoma The vessel is distended by tumour Htx eosin × 83

appearance and with the same concentration of tumour cells as the adult rats In 3 of the rats the tumour did not take No difference was found between the young and the adult rats concerning the manner of growth, extent, and metastasization of the tumours Lung deposits were seen in one of the rats (Table 1)

Serial Transplantation with Ascitic Fluid

Intraperitoneal injection of 1 ml of ascitic fluid, containing about 4 8 × 10⁶ cancer cells per ml, induced tumours in the abdominal cavity of all of 8 rats The growth and spread of the tumours did not differ from that described in the preceeding paragraph The abdominal cavity was found to contain 40–100 ml of ascitic fluid The tumour could be carried through 6 rat passages by intraperitoneal injec-

TABLE 2

Serial Passage of R R Sarcoma by Intraperitoneal Injection with Ascitic Fluid

Intraperitoneal passage No	1	2	3	4	5	6	7
Positive rats	8 8	3 3	8 8	3 3	2 2	7 3	0/3
Day of spontaneous death	9 11	8 15	6–12	6 8	8–16	8 10	
Cancer cells ml ascites	$4\ 8\times10^6$	$2\ 7\times10^6$	$4–12\times10^6$	$4–9\times10^6$	$20–23\times10^6$	$4–8\times10^6$	0



Fig 6

Diaphragm from a rat injected with R R sarcoma intraperitoneally
Observe extensive infiltration of diaphragmatic musculature Htx eosin × 43

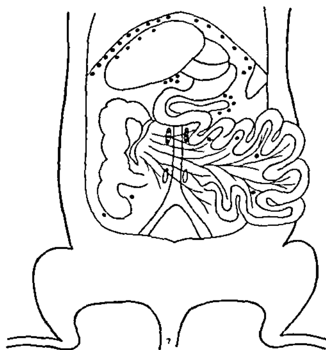


Fig 7

Diagram showing distribution of tumours in a rat injected intraperitoneally
with R R sarcoma

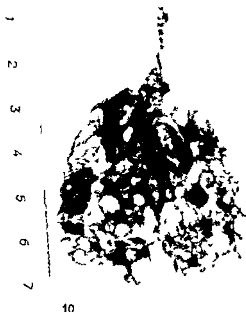


Fig 10
Lungs from a rat injected with R R sarcoma intravenously

the 13th and 18th day after the injection (Table 1) The remaining 2 rats were killed after one month Six of the rats showed extensive tumour deposits in the lungs (Fig 10) Histologically the tumours were mainly of the spindle cell sarcoma type and grew focally around the branches of the pulmonary arteries No metastases were found in the liver spleen or kidneys, but one of the rats had a secondary growth in the pancreas

Comparison between Rous Rat Sarcoma and Walker Carcinosarcoma Regarding Mode of Growth and Tendency to Metastasize

Certain similarities were found between the growth and spread of Rous rat sarcoma and of the Walker carcinosarcoma Intravenous, intraportal intramesenterial and intraperitoneal injections of corresponding doses of Walker suspension into Sprague Dawley rats produced tumours which closely resembled R R sarcomas concerning percentage of takes On intramuscular injection the Walker suspension also produced tumours with a greater tendency to metastasize in young rats than in adult animals

The R R and Walker tumours however differed in cytological and histological structures and also in mode of growth Intramuscular or subcutaneous injection with Walker suspension produced rapidly growing tumours which within 10 days assumed a mean size of 40 mm

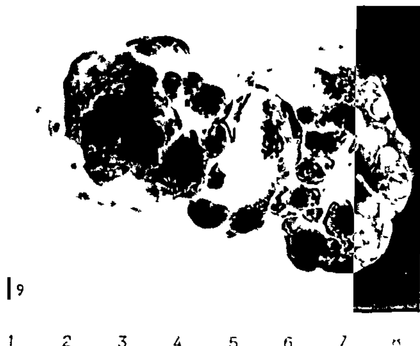


Fig 9
Liver from a rat injected with R R sarcoma intraportally

tion of 1 ml of ascitic fluid before it died (Table 2) Apart from some variation in the concentration of tumour cells in the ascitic fluid, no definite differences could be found between the different passages as regards manner of growth and tendency to metastasize

Intraportal Injection

Seven adult rats (weighing about 200 g) were injected intraportally with 0 1 ml of a suspension of R R sarcoma from the 5th rat passage Six of the rats died on the 12th 14th day and the 7th rat on the 30th day after injection (Table 1) In all of the rats the tumour had invaded and often largely replaced, the liver (Fig 9) The nodules were distributed more or less evenly in the liver parenchyma Histological examination showed that the tumour grew focally around the portal vessels as well as in bands in the liver sinusoids Though the spindle cells were numerous, the polymorphocellular form of sarcoma appeared to be predominant Many of the rats showed peritoneal carcinomatosis with haemorrhagic ascites Tumours were rarely seen at the site of injection The mediastinal lymph nodes showed only inflammatory changes No secondary growths occurred outside the abdominal cavity

Intravenous Injection

Seven adult rats (weighing about 200 g) were injected with 0 1 ml of a suspension from the 3rd rat passage Five of the rats died between

difference between the growth of the tumours after intramuscular and subcutaneous injection, respectively, are probably due to a better supply of nutrition in muscle tissue

After intraportal and intravenous injection tumours developed almost exclusively in the liver and the lungs, respectively, suggesting that most of the injected tumour cells are trapped in the capillary network of these organs All of the rats injected intraportally showed extensive growth in the liver and many of the animals inoculated intravenously had large metastases in the lungs The liver and the lungs evidently provide a favourable medium for the growth of R R sarcoma

The histological structure of the sarcoma did not vary with certainty with the site of the tumour The spindle cell type was most frequently represented in the infiltrating parts of the tumours The polymorphocellular type might possibly represent a commencing degeneration

As a rule, the intramuscular and subcutaneous R R sarcomas did not metastasize in adult rats On the other hand, the intraperitoneal tumours often showed metastases in the mediastinal lymph nodes, and sometimes though rarely, in the lungs The spread appeared to be largely lymphogenous The young rats injected intramuscularly with R R sarcoma on the other hand often showed lung metastases without coexistent deposits in the regional lymph nodes which suggests that in these cases the spread might have been haematogenous

The R R sarcoma grew better and metastasized more often in young than in adult rats injected intramuscularly In this respect the tumour resembled many other transplantable tumours

Though the rats used were not of an inbred strain the R R sarcoma developing on intraperitoneal injection grew almost uniformly in all of the animals The use of young rats instead of adult animals did not produce any improvement in the growth of the intraperitoneal tumours or in their tendency to metastasize Intraperitoneal injection was followed by the formation of abundant ascitic fluid containing tumour cells The concentration of the tumour cells, however, was relatively low and the tumour may not be regarded as an ascitic tumour Attempts to increase the percentage of cancer cells by passing the tumour successively by intraperitoneal inoculation and thereby convert the R R sarcoma to an ascitic form (*Klein & Klein* 1956) failed because the tumour for some unknown reasons died after 6 serial passages During these passages no changes were observed with certainty in the tendency of the tumour to metastasize

The local growth of transplantable tumours from early passages is usually less aggressive than from later passages with the results that the animals survive longer and hence, the tumour has more time to metastasize (*Ostenfeld* 1941) On adaptation to the host the tumour becomes more virulent, it kills the animal sooner and it therefore has less time to disseminate When injected intramuscularly or subcutaneously the R R sarcoma did not grow equally well as the Walker carcino-

× 40 mm and 30 mm × 40 mm, respectively It is true that after 14 days large, central, cystic necroses and widespread, superficial ulcerations occurred, but, generally speaking, the tumours continued to grow incessantly until the death of the animals, which often occurred within one month of injection In contrast to the experiments with R R sarcoma, no substantial difference was found between the growth of the tumours following intramuscular and subcutaneous injection

Metastases were invariably seen in the regional lymph nodes and occasionally also in more remote lymph nodes in rats injected with the Walker tumour by the subcutaneous or intramuscular route

On intraperitoneal injection the Walker tumour produced ascites and, like the R R sarcoma, it could be transferred serially by ascitic fluid but died after passage through 6 animals Animals with intraperitoneal tumours died between 7 and 20 days after inoculation The ascitic fluid contained 3–20 × 10^6 tumour cells per ml of ascitic fluid The Walker tumours appeared to have a less pronounced tendency to spread beyond the abdominal cavity than R R sarcomas Of 129 rats inoculated with the Walker tumour, only 3 showed unequivocal metastases in the anterior mediastinal lymph nodes and none of the animals had secondary growths in the lungs But not all of the animals were studied histologically, and only lymph nodes with suspected gross changes were studied histologically

DISCUSSION

In adult rats the transplants of R R sarcoma varied considerably with the site of injection

While the tumours developing after intraperitoneal injection killed a fairly large number of the rats within about 2 weeks, all of the rats injected subcutaneously or intramuscularly survived for at least 4 weeks The tumours developing after intramuscular and especially after subcutaneous injection, showed signs of regression in the second to third week The tumours also grew much better in animals injected intramesenterially than in those injected subcutaneously or intramuscularly The abdominal cavity thus appears to offer more favourable conditions for growth of the tumour than muscle or subcutis The tumour suspensions used for injection by the intramesenterial or intraperitoneal route were, as a rule, collected from somewhat later passages (3rd and 5th, respectively) than those used for intramuscular and subcutaneous injection (2nd passage), which might possibly explain the differences between the virulence of the tumours But, then again, the growth of the subcutaneous transplants did not differ from one passage to another (Fig 1), nor was growth inferior of tumours developing after intramesenterial injection with suspension from the 2nd passage to those induced by suspension from the 3rd tumour passage These observations argue against any change in virulence of the tumour The

gressively, and more frequently gave rise to metastases than the Rous rat sarcoma while the Rous rat sarcoma transplanted intraperitoneally gave rise to metastases outside the abdominal cavity more frequently than the Walker carcinosarcoma

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sarcoma, possibly because the R R sarcoma is younger The Walker sarcoma grew equally well when injected intramuscularly as subcutaneously, which suggests that it is less dependent on the local nutritional conditions than the R R sarcoma In the remaining sites the growth conditions were evidently very good with the doses used and thereby masked any differences between the two tumours With reference to what was said above, R R sarcoma might have been expected to metastasize more often than Walker carcinosarcoma, and on intraperitoneal inoculation it did However, no definite differences were found between the two types of tumour in respect of the duration of survival of the tumour-bearing animals, and hence it appears doubtful whether the difference in tendency to metastasize was due to different ages of the tumours On subcutaneous and intramuscular injection, on the other hand, the R R tumours did not metastasize as often as the Walker tumours This might be explained by the fact that the R R sarcoma started to regress early and allowed the cells no time to disseminate and give rise to secondary growths

SUMMARY

In the present investigation growth and spread of the transplanted Rous rat sarcoma by various routes of inoculation, was studied The sarcoma material used in the experiment originated from early rat passages

The Rous rat sarcoma transplanted intramuscularly and, above all, subcutaneously grew fairly slowly in adult rats and often showed signs of regression Metastases were rare When transplanted intramuscularly to young rats, the Rous rat sarcoma, on the other hand, grew better and gave rise to lung metastases

On intraperitoneal transplantation the tumour grew very rapidly, particularly in the greater omentum, diaphragmatic musculature and mesenterium of the small intestine, in both young and adult rats and killed most of the hosts within about 2 weeks The intraperitoneal tumours also give rise to abundant ascitic fluid containing tumour cells and could be serially transferred by this fluid in rats Lymph node metastases were often seen outside the abdominal cavity

Transplantation of Rous rat sarcoma to the mesenterial *radix* resulted in rapidly growing tumours occupying the larger part of the abdominal cavity

Intraportal and intravenous injection of a suspension of Rous rat sarcoma produced widespread tumour growths in the liver and lungs, respectively

On comparison between the growth and spread of Rous rat sarcoma and another rat tumour, Walker carcinosarcoma, several similarities were found but also some differences The Walker carcinosarcoma grew intramuscularly and subcutaneously much more rapidly and more ag-

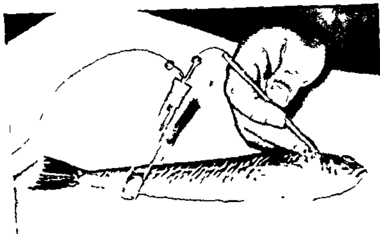


Fig 1
Extraction of weever toxin—"Micromethod"

a test tube was attached Extraction from living fish was difficult but had the advantage that toxin could be extracted from the same fish several times However, these methods were abandoned because of their scanty yield of toxin Instead, use was made of a method for the extraction of venom from dead fish, details of which will be given here

It is particularly important that the fish are put on ice immediately after being caught and immediately sent to the research centre Extraction should be made preferably immediately after arrival but if this is not practicable, the fish should be kept on ice until extraction can be performed

The apparatus used for the extraction of larger amounts of toxin (up to now from a total of 15 000 fish) consists of a 300 ml glass flask the neck of which is provided with a tight fitting rubber stopper in which two right angled glass tubes are inserted Rubber tubing fitted to one of the glass tubes leads to a suction

[illegible]

... test tube fitted with a *Larsten Schmidt* stopper, as shown in Fig 1

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THE SERUM AND TOXOID DEPARTMENT, STATENS SERUMINSTITUT
COPENHAGEN, DENMARK

WEEVERFISH TOXIN

Extraction Methods, Toxicity Determinations and Stability Examinations[1]

By

E. Skeie

Received 26. x. 61

No other poisonous fish than the weeverfish *(Trachinus draco)* seems to have been the subject of so many studies and of so much discussion. However, even so, the problems connected with the venom of this fish are far from solved. In the opinion of the writer, this is primarily due to the inadequate methods hitherto used for the extraction of the weever toxin. This, in turn, must be due to a lack of knowledge concerning the exact anatomical aspects of the venom apparatus and the stinging mechanism. As regards the majority of the studies in this field, insufficient access to weevers and absence of opportunities for studying the fish *in vivo* have doubtless also been contributory factors. In addition, most of the studies were carried out many years ago when the technical aids were not so well developed as they are to-day.

The writer has had the good fortune to work where large quantities of weeverfish could be procured at most times of the year. It has also been possible to study and work with living weevers in large aquariums. This has made it possible to work out an extraction method which can be regarded as ideal, in so far as one can speak of an ideal method in connection with such time-consuming work as extraction of weever toxin.

EXTRACTION METHODS

In the earliest published work (*Schmidt* 1874) the experimental animals were stung with the venom spines themselves. *Maretić* also used this method as recently as in 1957. *Phisalix* (1899) and *Briot* (1902) prepared extracts from macerated spines and surrounding tissue. *Evans* (1907) removed the toxin by drawing it out from the grooves of the spines using a sterile syringe.

The writer of the present work has tried these various methods, but without success, the amounts of toxin obtained being too crude and too small. In addition, efforts have been made to extract toxin from living weevers in aquariums by letting the fish sting a sponge, from which the toxin subsequently was removed by washing, and by letting the fish sting the opercular spines through a rubber membrane to which

[1] Part of the work was carried out at the *Physiological Laboratory of Denmark's Aquarium*. Financial assistance has been granted by the *P. Carl Petersen's Foundation* and the *Danish State Research Foundation (Statens almindelige Videnskabsfond)*.

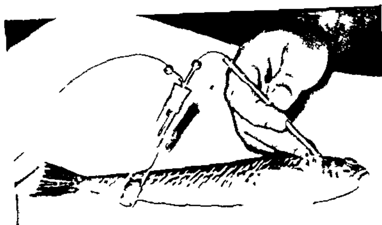


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It is particularly important that the fish are put on ice immediately after being caught and immediately sent to the research centre Extraction should be made preferably immediately after arrival but if this is not practicable the fish should be kept on ice until extraction can be performed

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tion when the apparatus is not in use During the whole extraction process the flask is placed in ice bath possibly containing small pieces of carbon dioxide ice Slime should be removed from the venom spines before suction It is necessary at intervals during extraction to rinse the suction point and the tubing with known amounts of physiological saline in order to avoid blockage of the system with glandular tissue

A "micromethod" has been used successfully for estimating the amounts of toxin in the individual fish or in the individual venom gland The large suction flask is substituted by a smaller test tube fitted with a Carsten Schmidt stopper as shown in Fig 1

The raw toxin consists of a greyish white solution containing copious sediment Immediately after extraction the solution is centrifuged in a water-cooled centrifuge at 10 000 r p m for ten minutes This results in the formation of a rather large amount of solid sediment consisting of cell remnants and a few unavoidable scales Sometimes there is a thin layer of froth on top this can easily be removed by filtration What is left is a completely clear solution of slightly increased viscosity—the "pure" toxin—with a pH of about neutral point (7 1)

TOXICITY DETERMINATIONS

Toxicity experiments carried out on animals (white mice and guinea pigs), and in tissue culture, will be discussed

Animal Experiments

A number of animal studies on weever toxin have been reported previously (*Schmidt* 1874, *Bottard* 1889, *Briot* 1902, *Evans* 1907, *de Marco* 1936, *Marelić* 1957, *Russell & Emery* 1960), but no actual quantitative titration of the toxin with determination of DML seems to have been made

Mice

Methods 0 2 ml of two fold dilutions of weever venom in physiological saline is injected into the tail veins of mice This dilution series gives a quite sharp endpoint since all of the mice die immediately or shortly after the injection of a concentration which is just sufficient to kill while animals receiving the subsequent lower concentrations are hardly affected At first up to six mice per concentration were used but because of the only slight variation observed it was found that the number of mice at each titration stage could be reduced to two without affecting the uniformity and reproduceability of the results The final reading is made after 24 hours The smallest amount of toxin which when injected intravenously into the tail veins of white mice weighing 16 to 18 g, kills 100 per cent of the animals within 24 hours is designated DML/100 The titre is stated as the reciprocal value of this figure

Results The following experiments illustrate the procedures used Toxin from 600 medium sized weevers (20 to 25 cm long) was extracted by means of the method described Physiological saline (40 ml) was added during extraction After centrifugation 60 ml of toxin were obtained (Fj 8) The results of toxin determination on mice are shown in Table 1

TABLE 1

Titration of Weever Toxin Fj 8 by Intravenous Injection into Mice

Toxin dilution	Observation after			
	5 minutes		24 hours	
	Mouse no		Mouse no	
	1	2	1	2
1 1024	Normal	Normal	Normal	Normal
1 512	Ill	Ill	Dead	Dead
1 256	Instant death	Instant death		

In this case 1 DML/100 was 0 2 ml of a toxin dilution 1 512 or approximately 0 0004 ml undiluted toxin The titre of the undiluted toxin is thus approximately 2500 DML/100 per ml The total number of DML/100 extracted from the 600 fish was about 150 000 DML/100, *i e* about 250 DML/100 per weever

Weever toxin has been extracted at different seasons of the year During extraction some fish are seen to contain more toxin than others, but the feature seems to be quite incidental, and it has not been possible to confirm the relationship between venomousness and season, alternatively spawning, mentioned in the literature (*Dunbar-Brunton* 1896, *Evans* 1907) During the production of 19 portions of weever toxin, comprising from 120 to 3500 fish per portion, the average toxin content per weever has been found to vary from 1066 right down to 6 DML/100, calculated according to the same principle as mentioned above The toxin concentration of the different extractions varied from 2560 to 640 DML/100 per ml

The largest amounts of toxin are to be found in the opercular glands because of their greater extent as compared with the glands in the first dorsal fin (this aspect will be reported elsewhere) This can be shown experimentally by means of the micromethod mentioned previously Extractions from the first dorsal fin of five weevers (21 to 25 cm long) showed no measurable concentrations of toxin when injected into white mice, whereas extractions from the opercular glands of the same fish showed a total content per weever of > 48 to < 96 DML/100

Guinea Pigs

The extent to which weever toxin may produce local skin reactions has been examined in guinea pigs

[illegible]

TABLE 2

Titration of Weever Toxin FJ 7 by Intracutaneous Injection into Depilated Guinea Pigs

Toxin dilution	Local reaction after	
	24 hours	48 hours
1 256	20 RI*	10 DR§
1 256	20 RI	10 DR
1 512	10 RI	–†
1 512	10 RI	
1 1024	–	–
1 1024	–	–
Phys saline	–	–
Phys saline	–	–

* R — redness I — infiltration
§ DR — diffuse redness
† DR — no reaction
The figures indicate the extent of the reaction in millimetres
The smallest reacting amount of toxin (DMR) is 0 2 ml of a dilution of 1 512 corresponding to about 2500 DMR/ml

Results Toxin Fj 7 was used for the experiment For the sake of comparison, titration was carried out simultaneously on mice Tables 2 and 3 give the results of these experiments

TABLE 3
Titration of Weever Toxin Fj 7 by Intravenous Injection into Mice

Toxin dilution	Observation after			
	5 minutes		24 hours	
	Mouse no		Mouse no	
	1	2	1	2
1 512	Normal	Normal	Normal	Normal
1 256	Dead	Moribund		Dead
1 128	Instant death	Instant death		

In this case 1 DML/100 is 0 2 ml of a toxin dilution 1 256 or approximately 0 0008 ml of the undiluted toxin, the titre of which is about 1250 DML/100 per ml

The experiment shows that in the course of 24 hours weever toxin can cause a skin reaction in guinea pigs which wanes after 48 hours The smallest amount of toxin (DMR) causing reaction is 0 2 ml of dilution 1 512 (0 0004 ml) This is half the amount of the lethal dose for mice, but is the same as the amount causing complete cell release for chick fibroblasts (see next section)

Tissue Culture[1]

It is well known that a number of toxins have a toxic effect on cell growth *in vitro* This fact has been utilized in recent years to determine the strength of snake, diphtheria, clostridium toxins, etc (among others, *Penso & Vicari* 1957, *Backhausz et al* 1957, *Edlinger & Dietel* 1959) It was thus natural to try the same methods with weever toxin Primarily it was hoped that it would be possible, by means of tissue culture, to find a method by which to measure toxin concentrations smaller than those determinable by animal experiments

Methods Use was made of various cell cultures of the monolayer type produced from

(1) Mixed tissues from 9 to 12 day old chick embryos chick fibroblasts
(2) Human embryonic kidney cells
(3) Rhesus monkey kidney cells
(4) Human amnion cells

The basic culture medium for all of the cell cultures consisted of Hank s balanced salt solution + 0 5 per cent lactalalbumin hydrolysate As regards Nos 1 2 and 3

1 The writer is grateful to Dr *Freundt Statens Seruminstitut* for the preparation of cultures

calf serum (2 per cent) was added and to No 4 horse serum (20 per cent) *Culture* was carried out in Carrel flasks containing 5 ml of culture medium and in some cases in test tubes containing 1 8 ml After a certain incubation period (varying from one cell type to the other, as regards the *fibroblasts 48 hours*) 0 2 ml of two fold dilutions of weever toxin was added to the tubes containing 1 8 ml of culture medium and correspondingly more toxin to the flasks containing 5 ml of culture medium *Physiological saline* was added to the control tubes The tubes were incubated for 24 hours at 37° C after which reading was carried out

Results Even with very small toxin concentrations, growth and cell changes could be seen microscopically, particularly the occurrence of multiple vacuoles in the cytoplasma and more profuse granulation These changes could be registered in several titration steps The extent of release of the cells from the wall of the tube was chosen as indicator for the effect of the toxin on the cells The borderline was found to be quite clear and well defined and was used as TCD 100 endpoint for the tissue cultures The effect of the toxin could be seen rather quickly—generally within two hours

In order to compare the methods for measuring the effect of weever toxin by intravenous injection on white mice weighing 16 to 18 g and by tissue culture (chick fibroblasts), parallel experiments were carried out with weever toxin F*I* 7 The potency of the toxin as determined on mice was the same as the results shown in Table 3 The results with tissue culture are shown in Table 4

TABLE 4
Titration of Weever Toxin (FI 7) on Chick Fibroblasts (KF 13)

Tube no	Toxin concentration	Extent of cell release*
1	Phys saline	—
2	1 4096	—
3	1 2048	—
4	1 1024	+
5	1 512	++++
6	1 256	++++
7	1 128	++++
8	1 64	++++
9	1 32	++++
10	1 16	++++
11	1 8	++++
12	1 4	++++
13	1 2	++++

* — = normal growth + = commencing cell release ++++ = total cell release

The TCD 100 for the tissue culture is 0 2 ml of toxin dilution 1 512 or 0 0004 ml undiluted toxin

It will be seen from the experiment that a well def[illegible]

[illegible] but which are the same as those just able to produce local skin reactions in guinea pigs

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other stabilizing agents such as albumin and enzyme inhibitors have been investigated but no suitable stabilizer could be found

Freeze drying has also been attempted but the results were unsatisfactory The actual lyophilization was tolerated, but after storage for a few days at 2–4°C in lyophilized state, the potency of the toxin had decreased considerably

At one stage it became clear that the loss of toxicity was greatest in batches originating from fish which had been a long time under transport to the laboratory Naturally it is impossible to carry out extraction of toxin under absolutely sterile conditions and not infrequently growth in toxin preparations could be demonstrated by culture on agar and blood agar plates, even immediately after extraction The bacteria have not been classified, but growth is due probably to contamination by common bacteria during the extraction However, it cannot be precluded that the toxin may have contained also marine bacteria with a low life optimum causing proteolysis even at very low temperatures In order to avoid possible bacterial growth, the addition of various antibiotics was tried, but without satisfactory result

Attempts to sterilize the crude toxin extract by filtration were made, and this, combined with the addition of glycerin, appeared to solve the storage problem

The procedure adopted in consequence of these prolonged experiments is briefly as follows – Immediately after extraction and centrifugation as described above the toxin is filtered under sterile condi [illegible]

[illegible] unchanged for more than two years

SUMMARY

The various methods for the extraction of weever toxin are reviewed

A report is given of potency determinations carried out on white mice and guinea pigs and in tissue culture

Experiments regarding the storage of the toxin are mentioned, and a method is described by which the toxin can be stored for more than two years without any decrease in toxicity being involved

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Brist M [illegible]

Dunbar B [illegible]

In some experiments human embryonic kidney cells, rhesus monkey kidney cells, and human amnion cells were used instead of chick fibroblasts The human kidney cells showed the same sensitivity as the fibroblasts with sharp endpoint, while the toxin effect was much weaker, there was no characteristic endpoint when rhesus monkey kidney cells and human amnion cells were used

The amnion culture medium contains 20 per cent horse serum, while 2 per cent calf serum is added to the fibroblasts Theoretically, it might be anticipated that the horse serum would have a neutralizing effect on the toxin, and therefore calf serum was substituted in one experiment, but the result was the same It takes about five days for the monkey kidney cells to form a confluent cell layer, while the fibroblasts require only two days It is possible that the culture with the slowest metabolism—the monkey kidney cells—needs longer toxin effect than, for example, the fibroblasts, and that the toxin is denatured in the incubator before the adequate time has elapsed This aspect has not yet been examined

The primary object of using tissue culture in these experiments was to find a sensitive method by which to measure the weever toxin, without investigating further the more detailed cytotoxic cell changes Judging by the experiments with the chick fibroblasts, this aim has been achieved, since the results were uniform and reproduceable

STABILITY INVESTIGATIONS

If the weever toxin is to be used over a long period, its storage presents a great difficulty, since it has proved to be very labile Earlier investigators (*Phisalix* 1899, *Briot* 1902, *de Marco* 1936) have chiefly used glycerin extracts produced by maceration of venom spines and surrounding tissue *Evans* (1907) dried the toxin when it had been extracted with a syringe These methods have also been attempted in the present study, but even after storage for short periods at 2–4°C the loss in toxicity was marked and it was necessary to find other means of storage

Hence the effect of various factors on the toxin stability has been examined by the writer

The temperature was found to be of significance Storage at low temperatures or freezing of the untreated toxin increased the stability to some extent, but even at −60°C a gradual loss in stability was noted When the frozen preparations were allowed to thaw, sedimentation occurred, and spectrophotometry showed a decrease in the protein maximum at 278 mμ

Storage at hydrogen ion concentrations varying from pH 4 2 to 8 7 was examined at −60°C and +4°C The toxin was found to be most stable about neutral point at the lowest temperature

Storage in different glycerin concentrations has been tried and also

other stabilizing agents such as albumin and enzyme inhibitors have been investigated, but no suitable stabilizer could be found

Freeze drying has also been attempted but the results were unsatisfactory The actual lyophilization was tolerated, but after storage for a few days at 2–4°C in lyophilized state, the potency of the toxin had decreased considerably

At one stage it became clear that the loss of toxicity was greatest in batches originating from fish which had been a long time under transport to the laboratory Naturally it is impossible to carry out extraction of toxin under absolutely sterile conditions and not infrequently growth in toxin preparations could be demonstrated by culture on agar and blood agar plates, even immediately after extraction The bacteria have not been classified, but growth is due probably to contamination by common bacteria during the extraction However, it cannot be precluded that the toxin may have contained also marine bacteria with a low life optimum causing proteolysis even at very low temperatures In order to avoid possible bacterial growth, the addition of various antibiotics was tried but without satisfactory result

Attempts to sterilize the crude toxin extract by filtration were made, and this, combined with the addition of glycerin, appeared to solve the storage problem

The procedure adopted in consequence of these prolonged experiments is briefly as follows – Immediately after extraction and centrifugation as described above, the toxin is filtered under sterile conditions through Seitz FKS pads under pressure Fifteen per cent glycerin is added the toxin is frozen quickly in a mixture of alcohol and carbon dioxide ice, and stored at –60°C By this means the toxicity has remained unchanged for more than two years

SUMMARY

The various methods for the extraction of weever toxin are reviewed

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FROM THE UNIVERSITY INSTITUTE OF HUMAN GENETICS, COPENHAGEN (CHIEF: PROFESSOR TAGE KEMP, M.D.) AND THE UNIVERSITY INSTITUTE OF PATHOLOGICAL ANATOMY, COPENHAGEN (CHIEF: PROFESSOR J. ENGELBRETH-HOLM†)

METACHROMASIA IN THE DERMAL CONNECTIVE TISSUE FROM PATIENTS WITH HAEMOPHILIA

By

KNUD ERIK SJØLIN

Received 5.i.62

In an earlier communication a metachromatically staining reaction in the dermal connective tissue from two haemophilic patients was demonstrated (*Sjølin* 1961). The biopsies were stained with aqueous toluidine blue, which gave the connective tissue a purple colour. This is not seen in skin tissue from normal persons.

Since then the author has examined 19 skin biopsies from 18 haemophilic patients.

METHODS

The biopsies were taken with a cutting punch on the back of the [illegible] [illegible]

The tissue samples were fixed in a 4 per cent solution of basic lead acetate, and after the usual histological preparation stained for half an ho[illegible]

RESULTS

The results of this investigation are summarized in Table 1. The numbering of the patients and the families refers to an earlier work (*Sjølin* 1960). The degree of the metachromasia in the connective tissue in derma and subcutis is expressed as: Weak, distinct and strong. In tissue samples with questionable metachromasia the designation none is used.

Among the 19 biopsies from 18 haemophilic patients metachromatically staining reaction of the connective tissue was demonstrated in

The histological slides used in this work were prepared in The Laboratory for Connective Tissue Research, Department of Anatomy, University of Copenhagen. Professor *Asboe-Hansens* readiness to help with this problem is greatly appreciated.

12 (Table 1) The metachromasia was strong in 6 cases, distinct in 3 and weak in 3 cases (Fig 1) There was not any metachromatically staining reaction in 7 biopsies from 6 out of 18 patients, *i e* one third of all of the examined patients Metachromasia was never found in the tissue samples fixed in formalin solution

TABLE 1

Family	Patient	Age of the patient in years	Deficiency in clotting system	Metachromasia
2	12	29	Antihaemophilic factor (AHF)	Strong
2	14	20	"	Strong
2	15	17	"	Distinct
66	2	12	"	None
	"	3 weeks later	"	None
68	1	13	"	*Distinct*
72	1	6	"	Weak
4	10	5	*Christmas factor*	*Strong*
51	3	32	"	Weak
97	2	1	"	Strong
106	1	3	Plasma thromboplastin antecedent	Strong
1	4	42	AHF + Christmas factor (combined haemophilia)	None
1	9	1	"	Strong
75	1	5	AHF + freezing/serum factor (combined haemophilia)	Weak
108	1	16	"	*None*
51	4	18	Hageman like factor	Distinct
90	1	4	"	None
70	1	10	Thromboplastin inhibitor	None
		1	Not classified	None

The present material comprised all known types of haemophilic clotting defects Among the biopsies from 6 patients with AHF (factor VIII) deficiency 5 presented metachromasia of varying degree

Metachromasia was demonstrated in all of the biopsies from 3 patients with Christmas disease (lack of factor IX) In the biopsy from a patient with deficiency of plasma thromboplastin antecedent (factor XI) metachromasia was demonstrated

Two patients with combined haemophilia (simultaneous deficiency of AHF (factor VIII) and Christmas factor (factor IX)) belonging to *the same family were examined In one of these metachromasia was* demonstrated

In the biopsy from one of two patients with deficiency of *AHF* + "freezing/serum" factor (combined haemophilia Deficiency of factor VIII + IX) metachromasia was found

Two patients with *Hageman like defect (factor XII)* were examined (*Sjolin* 1959) One of these biopsies showed metachromasia

In the biopsy from a patient with thromboplastin inhibitor (*Sjolin* 1960) metachromasia was not demonstrable One patient with haemo-



Fig 1
Metachromatical staining reaction in a skin biopsy from a patient with haemophilia

philia died before he had his defect in the plasma clotting system classified The biopsy from this patient did not reveal metachromasia

The metachromatical staining reaction in the biopsies gradually faded away after 18 months Apart from the metachromasia the biopsies did not reveal any pathological changes

COMPLICATIONS

One patient (Family 2 no 14) removed the sutures from the biopsy place a few days after the operation This procedure was followed by an ulceration of the skin with oozing bleeding The ulceration persisted for several months

COMMENTS

The previous demonstration of metachromasia in the dermal connective tissue in patients with haemophilic disease was confirmed The metachromatical staining reaction was demonstrated in 12 out of 18 patients All groups of haemophilia were included among the 12 patients AHF deficiency (factor VIII) lack of Christmas factor (factor IX) combined haemophilia (factor VIII + IX) PTA deficiency (factor XI) and Hageman like deficiency

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metachromatically staining reaction was weak. Thus the metachromasia does not seem to depend on a certain *clotting defect*.

The age of the patients ranged from one to 42 years. Seven patients were 16 years old or more. Five in this group showed metachromasia. Among the 11 younger patients 7 showed metachromasia. Thus the metachromatically staining reaction does not depend on the age of the patients.

Only one of the patients (Family 1, no. 9) was in hospital on account of a bleeding episode, when the biopsy was taken. None of the patients were suffering from other diseases than haemophilia.

In the present material the clinical appearance of the haemophilic disease seems to be uniform in the groups with and without metachromasia.

It is known, that the bleeding tendency and the clotting defect in haemophilia varies (*Sjolin* 1958, *Sjolin* 1960), although these two phenomena not always seem to be correlated. One of the questions to be solved is, whether the metachromatically staining reaction in patients with haemophilia varies.

Metachromatically staining reaction with toluidine blue is given by acid mucopolysaccharides. It is known that sulphonated acid mucopolysaccharides inhibit the clotting of blood (*Jorpes* 1936, *Bergstrom* 1936, *Astrup* 1944, *Piper* 1945). Among the sulphonated mucopolysaccharides is for instance heparin *(Jorpes)*.

The experiments of *Lowenburg & Rubenstone* (1918) indicated the presence of a clotting inhibitor in extract from the liver and thyroid gland in a haemophilic patient. *Sjolin & Astrup* (1958) have so far demonstrated a *thromboplastin inhibitor in the synovial membrane* and the fibrous capsular tissue of the knee joint from a haemophilic patient with AHF deficiency (Factor VIII). This inhibitor could, like *heparin, also be extracted* with potassium cyanate (*Snellman, Jensen & Sylven* 1948).

The pathogenesis of haemophilia is still obscure in as much as a discrepancy is often observed between the clotting defect and bleeding tendency. Therefore it seems reasonable to consider the influence of other possible factors on the haemostatic mechanism. The results presented here and earlier published investigations indicate, that a clotting inhibitor may be present in the ground substance of the connective tissue in some haemophilic patients.

SUMMARY

Skin biopsies were taken from 18 otherwise healthy haemophilic patients. In 12 of the *cases metachromatically staining* reaction with toluidine blue was demonstrated. This abnormal staining reaction did not depend apparently upon a particular clotting defect or the age of the patient. Metachromasia with toluidine blue is given by acid muco-

polysaccharides Among these the sulphonated ones are known to inhibit the coagulation of blood On the basis of the present and earlier investigations it seems reasonable to suppose, that a tissue inhibitor may be of importance in the bleeding tendency in the haemophilic diseases

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media contained 100 units of penicillin and 100 μg of streptomycin per ml In a few experiments monolayer cultures of kidney epithelial cells from humans and from calves were also employed These cultures were prepared by trypsinization of small pieces from the cortex of the kidney and were grown and maintained in the same manner as cultures of plexus choroideus

Virus.—Strain K483 of visna virus was used for inoculation of the cultures It had undergone 36 serial passages in sheep tissue culture cells and had a titer of 10^{-6} $TCID_{50}$ per 0 1 ml Before use the infectious tissue culture fluid was centrifuged in an International centrifuge at 1500 RPM for 10 minutes and was subsequently stored at −50° C The same batch of virus was employed in all experiments

Assay of virus.—Roller tube cultures of sheep cells were used for virus assay (6) In virus titrations serial tenfold dilutions were inoculated in 0 1 ml amounts into roller tubes using three tubes per dilution In neutralization tests 0 2 ml of each serum virus mixture were inoculated into each of three culture tubes The tubes were incubated in roller drum at 37° C and were examined for cytopathic changes at 3–4 day intervals for 35 days

Neutralization tests.—The neutralization tests were carried out as described previously (5) Serial twofold dilutions of the sera were made in Hanks salt solution and were mixed with an equal volume of a virus dilution containing approximately 100 $TCID_{50}$ per 0 1 ml The mixtures were incubated in an ice bath for 48 hours and were then assayed for virus activity as described above The highest serum dilution which inhibited virus activity in at least one of the culture tubes was considered the neutralizing titer A normal sheep serum found to be free from visna neutralizing antibodies and a reference visna antiserum were used as controls in all the tests

EXPERIMENTS AND RESULTS

Inoculation of cultures with a large amount of visna virus.—Four to five culture tubes from each of the various animals were inoculated with 0 1 ml of undiluted virus and were then incubated with 1 ml of maintenance medium in roller drum at 37° Approximately the same number of uninoculated cultures served as controls Two days after inoculation the cultures were washed five to six times with medium 199 and the fluids of the last washing were saved for later titration of virus The cultures were then incubated further for various periods of time During the first two weeks of incubation, the maintenance medium was changed every four to five days and after that every ten to twenty days The removed media were harvested and stored at −50° and later assayed for visna virus by titration in sheep cultures The cultures from the various animals were examined in the microscope for cytopathic changes at intervals ranging from two to seven days At the end of each experiment varying from 20 days to three months after inoculation, the cultures were fixed and stained with Giemsa

Figure 1 shows growth curves of visna virus in primary cultures of choroid plexus from the various animal species In calf cultures, the virus titer increased to $10^{-5.5}$ in about ten days In the cultures from the other species the virus titer did not rise above approximately 10^{-3} but virus activity persisted in the culture fluid for as long as two to three months The shorter persistence in cultures from cat was probably due to poorer condition of the cells employed in this experiment

Cytopathic changes similar to those seen in sheep cultures infected with visna virus (5), were observed in the infected calf cultures where most of the cells finally degenerated and fell off the glass In Giemsa

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GROWTH OF VISNA VIRUS IN PRIMARY TISSUE CULTURES FROM VARIOUS ANIMAL SPECIES[1]

By

HALLDOR THORMAR and BERGTHORA SIGURDARDOTTIR

Received 2 x 61

Visna is a virus infection of the central nervous system of sheep causing pleocytosis in the cerebrospinal fluid and often progressive paralysis leading to death (3) The characteristic lesion both in natural and transmitted cases is demyelination of the white matter of the brain and the spinal chord, apparently resembling lesions in the human demyelinating diseases (3, 4)

In the field, visna has been found to occur only in sheep, and attempts to transmit the disease to various other animal species by intracerebral injection of infectious material have so far been unsuccessful (3)

Visna virus has been propagated in tissue cultures from sheep, first in explants of choroid plexus or of white matter from the brain (5), and later in cultures of kidney and liver cells (7), and in explants of lung spleen, and salivary gland (8) Sheep inoculated intracerebrally with tissue culture passages of the virus have been found to develop signs and anatomical lesions characteristic of visna (5)

The present paper will describe attempts to infect tissue cultures from humans and from various domestic and laboratory animals with visna virus The results of preliminary neutralization tests with sera of various mammalian species against visna virus will also be reported

MATERIAL AND METHODS

Tissue culture —Primary cultures of choroid plexus from six to nine week old human embryos and from newborn to about four day old calves pigs cats dogs and guinea pigs were used in the present work Pieces of the minced tissue were explanted in a clot of chicken plasma (Difco) in roller tubes as has been described previously for cultures of plexus choroideus from sheep (5) The human cultures were grown in medium 199 with 20 per cent placental cord serum and the animal cultures were grown in medium 199 with 20 per cent calf serum When extensive outgrowth of cells had formed in the tubes usually 7 to 10 days after explantation the cultures were washed three times with Hanks salt solution and were then inoculated with virus Medium 199 was used as maintenance medium except in some of the experiments with human cultures where 2 per cent placental cord serum was added This had previously been found to be free from inhibitors of visna virus All

1 Supported by a grant (No B 2425) from the Department of Health Education and Welfare U S Public Heatlh Service

Passage of virus—In a few experiments it was attempted to make serial passages of visna virus in cultures of choroid plexus from calves human embryos pigs, and guinea pigs One tenth ml of fluid medium from inoculated cultures showing the highest virus titer by titration in sheep cells (cf Fig 1) was passed into tubes with fresh cultures Ten and four passages respectively were carried out in cultures from calves and human embryos, while only one passage was made in cultures from pigs and guinea pigs The highest virus titers obtained in each passage are listed in Table 1 It can be seen that maximum virus titers ranging from 10[illegible] to $10^{-6.5}$ were obtained in passages in calf cultures and in the human cultures titers of $10^{[illegible]}$ and $10^{-3.5}$ were obtained In the latter each passage was observed for a period of two months and an approximately constant virus titer was found to persist in the cultures to the end of this period in spite of repeated renewal of the culture fluid A very low infectivity was found after passing the virus once in cultures from pigs and the infectivity was completely lost in the first serial passage in guinea pig cultures

TABLE 1

Infectivity Titers of Serial Passages of Visna Virus in Tissue Cultures from Various Animal Species

Animal	Highest virus titer obtained in each passage				
	1 pass	2 pass	3 pass	4 pass	10 pass
Calf	$10^{-6.5}$ (7d)	$10^{-6.0}$ (8d)		$10^{-6.[illegible]}$ (9d)	$10^{[illegible]}$
Man	$10^{-[illegible].5}$ (21d)	$10^{-3.5}$ (17d)	$10^{-2.5}$ (23d)	$10^{[illegible]}$ (14d)	
Pig	$10^{-0.7}$ (43d)			–	
Guinea pig	not demonstrable				

Numbers in brackets indicate the time in days elapsed since the virus was passed

Cytopathic changes resulting in complete destruction of the cells developed in calf cultures inoculated with first to tenth virus passage In Giemsa stained human cultures only a few multinuclear giant cells were seen Such cells were not observed in cultures from pigs and guinea pigs

Neutralization tests—A few sera from various animal species were tested for neutralizing substances against visna virus Some of these sera derived from the animals which were sacrificed in order to obtain tissue cultures for experiments with cultivation of the virus In addition a number of human sera[1] and of sera from animals of various ages were examined Sera from patients with multiple sclerosis were also included All of the sera were tested without previous heating but some of the bovine sera were tested also after heating to 50° for 30 minutes

The results of the neutralization tests are shown in Table 2 Bovine sera both from cows and from newborn calves showed a remarkably high degree of neutralization of visna virus that was not lost upon heating

1 Kindly supplied by the *State Hospital* and the *Municipal Hospital in Reykjavik*

stained calf cultures, giant cells were seen of the type which has been found to be characteristic of sheep plexus cultures infected with visna virus (5). Inoculated kidney cell cultures from calf also showed cytopathic changes and increased virus titer in the fluid

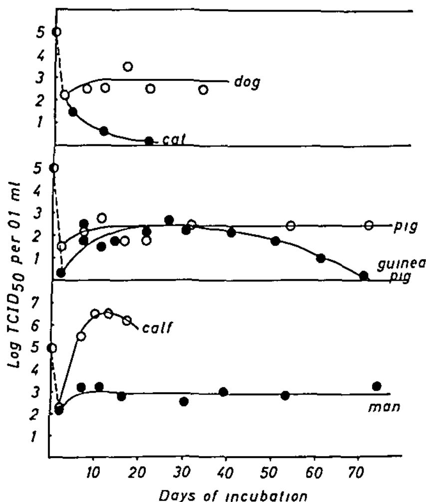


Fig 1

Growth curves of visna virus in tissue cultures from various animal species

In cultures of plexus choroideus from the other animals studied, cytopathic changes were not observed except in the human fetal cultures where stellate giant cells were sometimes seen Giemsa stained cultures, however, showed a few multinuclear giant cells, usually appearing together in small groups The multinuclear cells were found in greatest number in cultures from human embryos— both of choroid plexus and kidney cells—but most of the cells still looked normal and like those in uninfected cultures

Virus was not detected in the uninoculated control cultures from any of the animals included in the present study, nor did the control cultures show any multinuclear giant cells

Passage of virus.—In a few experiments it was attempted to make serial passages of visna virus in cultures of choroid plexus from calves, human embryos, pigs and guinea pigs. One tenth ml of fluid medium from inoculated cultures, showing the highest virus titer by titration in sheep cells (cf. Fig. 1), was passed into tubes with fresh cultures. Ten and four passages, respectively, were carried out in cultures from calves and human embryos, while only one passage was made in cultures from pigs and guinea pigs. The highest virus titers obtained in each passage are listed in Table 1. It can be seen that maximum virus titers ranging from $10^{-[illegible]5}$ to $10^{-6.5}$ were obtained in passages in calf cultures, and in the human cultures titers of $10^{-[illegible]}$ and $10^{-3.5}$ were obtained. In the latter each passage was observed for a period of two months and an approximately constant virus titer was found to persist in the cultures to the end of this period in spite of repeated renewal of the culture fluid. A very low infectivity was found after passing the virus once in cultures from pigs, and the infectivity was completely lost in the first serial passage in guinea pig cultures.

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Man	$10^{-2.5}$ (21d)	$10^{-3.5}$ (17d)	$10^{-[illegible].5}$ (23d)	$10^{-[illegible].5}$ (14d)	–
Pig	10^{-0} (43d)			–	–
Guinea pig	not demonstrable				

Numbers in brackets indicate the time in days elapsed since the virus was passed.

Cytopathic changes resulting in complete destruction of the cells developed in calf cultures inoculated with first to tenth virus passage. In Giemsa stained human cultures only a few multinuclear giant cells were seen. Such cells were not observed in cultures from pigs and guinea pigs.

Neutralization tests.—A few sera from various animal species were tested for neutralizing substances against visna virus. Some of these sera derived from the animals which were sacrificed in order to obtain tissue cultures for experiments with cultivation of the virus. In addition a number of human sera and of sera from animals of various ages were examined. Sera from patients with m[illegible] included[1] [illegible] of the bov[illegible]

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to 56° for 30 minutes. A few of the human sera showed neutralization in the lowest dilutions, and some of the sera from patients with multiple sclerosis neutralized the virus in dilutions up to 1:16. All of the other sera tested were either completely negative under the conditions employed, or had only a slight inhibiting effect in the lowest dilution.

TABLE 2
Neutralization Tests with Visna Virus and Sera from Various Animals

Animal	Number of sera tested	Negative in dil 1:4	Number of sera positive in dilution						
			1:4	1:8	1:16	1:32	1:64	1:128	>1:256
Man Normal	14	3	9	2	–	–		–	–
MS*	16	5	6	2	3	–	–	–	–
Bovine Cow	21	–	3		2	8	3§	3	2
Calf	5	–	1			–	1§		3
Horse	5	5	–	–		–		–	–
Rabbit	9	9	–	–	–	–	–	–	–
Guinea pig	17	15	2	–	–	–		–	–
Dog	5	3	2	–	–	–		–	–
Cat	8	4	4	–		–		–	–

* Patients with multiple sclerosis
§ These sera were tested also after heating to 56° C for 30 min. without showing change in titer.

DISCUSSION

The present study was undertaken in order to obtain information about the in vitro host-range of visna virus. Of the six species studied, only tissue cultures from calves showed a clear evidence of virus propagation. The rise in virus titer to $10^{6.5}$ in the inoculated cultures (cf. Fig. 1) represents approximately a 70-fold increase over the original inoculum, and the maximum titer of $10^{5.5}$ to 10^{6} obtained through ten serial passages indicates a continued multiplication of the virus in calf cells. The viral propagation in cultures from calfs was accompanied by distinct cytopathic changes and cell degeneration.

In tissue cultures from the other five species studied, the virus titer did not rise to the titer of the original inoculum. On the other hand, in spite of repeated renewal of the fluid medium, virus activity persisted in the cultures for as long as two to three months at a level of about one per cent of the inoculum. In view of the previous finding (6) that visna virus was inactivated in tissue culture fluid at 37° at a rate of about one log unit in 12 hours, the persistence of viral activity in the cultures indicates propagation of virus sufficient to overcome the loss caused by heat inactivation. Another interpretation is possible, namely that viral inactivation is slower in cell cultures than in cell free nutritional fluid, as found for influenza virus and Newcastle disease virus (2), and that the virus particles of the original inoculum are gradually liberated from the surface of the cells. This interpretation, however, is made less likely by the finding that virus activity disappeared

in a few days—and did not reappear—in cultures of human amniotic cells HeLa cells and L cells inoculated with a large amount of visna virus and remaining in good condition for over a month (8) The occurrence of multinuclear giant cells in cultures where viral activity was found to persist over a long period of time indicates that actually a few cells became infected and produced virus, while most of the cells were not affected

The finding that visna virus could be passed serially in cultures of human embryos supports the conclusion that it was propagated by the human cells The lower titer obtained after passing the virus in cultures from pigs might be due to poorer condition of the pig cultures in the present experiments rather than to less susceptibility of pig than of human cells The failure of passing the virus in guinea pig cultures is on the other hand more likely due to an inferior ability of guinea pig cells to propagate the virus since these cultures appeared healthy throughout the experimental period

The significance of the finding that bovine sera contained neutralizing substances against visna virus often in high titer is uncertain In view of the ability of calf cells to propagate the virus it does not seem unlikely that cattle may be natural hosts of visna virus even though they do not show signs of illness However, neutralizing substances were found not only in sera of cows in the immediate vicinity of the laboratory but also in cow sera from areas where visna is not

[illegible] remains that they represent either bovine viral antibodies which cross react with visna virus or that they are non specific heat stable inhibitors (1) The neutralizing substances have been found in sera from newborn calves but as yet, we do not know whether they disappear from the sera of the calves during the earliest months of life to reappear later It is not known either, whether they are found in the gamma globulin fraction of the serum Until they have been studied with respect to these two characteristics which are considered criteria of true antibodies a further discussion of the significance of the neutralizing substances would appear infeasible

Because of the apparent similarity between visna and the human demyelinating diseases (3 4) neutralization tests with sera from patients with multiple sclerosis were included in the present study as a pilot experiment Neutralizing substances against visna virus were found in sera from the patients in dilutions as high as 1 16 However, neutralizing activity was also found in normal human sera and on the basis of this preliminary study it cannot be concluded whether there is a significant difference between sera from patients with multiple sclerosis and normal human sera with respect to the ability to neutralize visna virus Also it is unknown whether the substances are heat stable Further study of human sera on a larger scale is in progress

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Calf	5		1		–	–	1§	–	3
Horse	5	5	–	–	–	–			–
Rabbit	9	9	–	–	–	–		–	–
Guinea pig	17	15	2		–	–		–	–
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FROM THE NATIONAL BACTERIOLOGICAL LABORATORY STOCKHOLM AND THE RHEUMATOLOGY DEPARTMENT KAROLINSKA SJUKHUSET STOCKHOLM

INHIBITION OF ANTIBODY FORMATION BY PREDNISOLONE

LOCATION OF A SHORT SENSITIVE PERIOD

By

KAARE BERGLUND

Received 29 xi 61

It has been shown that the early phase of the immune hemolysin response in rats can be inhibited if crystalline cortisone acetate is administered intramuscularly on the two days preceding antigen injection (*Berglund* 1956a) Prolongation of the pre treatment period up to seven days did not significantly increase this effect Pre treatment with cortisone for seven days was ineffective if discontinued the day preceding antigen injection (day 1) Furthermore administration of cortisone begun on the day of antigen injection had no significant effect

These results suggest that cortisone must produce some alteration in the animal *before* the introduction of antigen in order to affect the early phase of the hemolysin response The observation that omission of treatment on day 1 lead to a loss of effect of cortisone indicated that this injury was rapidly reversible However the protracted absorption of crystalline cortisone from the injection sites—leading to a cumulation—implied considerable uncertainty with regard to interpretation of time relations *Berglund* 1956a)

More accurate information about the early cortisone sensitive period could probably be obtained by giving frequent injections of a solution of cortisone which is rapidly absorbed and eliminated (*Bradlow et al* 1954) Unpublished experiments indicate that not only are large doses required but it may also be
alcohol which is difficult
which have the qualitative
isregarding
influence on water and electrolyte metabolism prednisolone has some salts which are easily dissolved in water As prednisolone has been shown to have a suppressing effect on antibody formation in mice almost equivalent to that of cortisone (*Foley et al* 1957) it was decided to use one of these salts

In the following experiments an aqueous solution of prednisolone disodium 21 phosphate was used to study some time factors conditioning its effect on the early hemolysin response in the mouse

SUMMARY

The ability of visna virus to grow in tissue cultures from various animal species was studied. The virus was found to be propagated through at least ten serial passages in cultures from calves, where it produced cytopathic changes resulting in complete destruction of the cells. In inoculated cultures from human embryos, pigs, dogs, cats and guinea pigs a low virus level persisted for a period of one to three months. A few multinuclear giant cells were seen in the cultures, but most of the cells looked normal at the end of this period. The virus was passed through four serial passages in human cultures and through one passage in pig cultures. In cultures from guinea pigs the virus activity could not be passed.

Visna virus was found to be neutralized by sera from calves and cows in dilutions up to 1:256 or higher, and by human sera in dilutions up to 1:16. Sera from five other species tested gave either completely negative results or had only a slight inhibiting effect in the lowest dilution (1:4).

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INHIBITION OF ANTIBODY FORMATION BY PREDNISOLONE
LOCATION OF A SHORT SENSITIVE PERIOD

By

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It has been shown that the early phase of the immune hemolysin response in rats can be inhibited if crystalline cortisone acetate is administered intramuscularly on the two days preceding antigen injection (*Berglund* 1956a). Prolongation of the pre-treatment period up to seven days did not significantly increase this effect. Pre-treatment with cortisone for seven days was ineffective if discontinued the day preceding antigen injection (day —1). Furthermore, administration of cortisone begun on the day of antigen injection had no significant effect.

These results suggest that cortisone must produce some alteration in the animal *before* the introduction of antigen in order to affect the early phase of the hemolysin response. The observation that omission of treatment on day —1 lead to a loss of effect of cortisone indicated that this injury was rapidly reversible. However, the protracted absorption of crystalline cortisone from the injection sites—leading to a cumulation—implied considerable uncertainty with regard to interpretation of time relations (*Berglund* 1956a).

More accurate information about the early cortisone sensitive period could probably be obtained by giving frequent injections of a solution of cortisone which is rapidly absorbed and eliminated (*Bradlow et al.* 1954). Unpublished experiments indicate that not only are large doses required but it may also be necessary to use a concentration of cortisone alcohol which is difficult to obtain. Among 17-OH corticosteroids which have the qualitative biologic effects of cortisone disregarding influence on water and electrolyte metabolism, prednisolone has some salts which are easily dissolved in water. As prednisolone has been shown to have a suppressing effect on antibody formation in mice almost equivalent to that of cortisone (*Foley et al.* 1957), it was decided to use one of these salts.

In the following experiments an aqueous solution of prednisolone disodium 21-phosphate was used to study some time factors conditioning its effect on the early hemolysin response in the mouse.

SUMMARY

The ability of visna virus to grow in tissue cultures from various animal species was studied. The virus was found to be propagated through at least ten serial passages in cultures from calves where it produced cytopathic changes resulting in complete destruction of the cells. In inoculated cultures from human embryos, pigs, dogs, cats and guinea pigs a low virus level persisted for a period of one to three months. A few multinuclear giant cells were seen in the cultures but most of the cells looked normal at the end of this period. The virus was passed through four serial passages in human cultures, and through one passage in pig cultures. In cultures from guinea pigs the virus activity could not be passed.

Visna virus was found to be neutralized by sera from calves and cows in dilutions up to 1:256 or higher, and by human sera in dilutions up to 1:16. Sera from five other species tested gave either completely negative results or had only a slight inhibiting effect in the lowest dilution (1:4).

ACKNOWLEDGEMENT

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on day —1 a dose of 1 0 mg and on day 0 1 25 mg[3], i e a total, daily dose of 10 mg

Group 5 received intramuscular injections of saline following the schedule of groups 2 and 4, and served as control

TABLE 1

Exper 1 Effect of two Dose Levels of Prednisolone (3 and 10 mg per Day) and of two Different Lengths of Its Administration (day —1 and day —1 + day 0) on the Hemolysin Response §*

Treatment	Hemolysin titer					
	Treated day 1			Treated day 1 and day 0		
	Group no	n	Mean ± S E M	Group no	n	Mean ± S E M
Prednisolone 3 mg day	1	10	5 11 ± 0 29	2	9	4 64 ± 0 63
Prednisolone 10 mg/day	3	10	4 06 ± 0 42	4	9	4 42 ± 0 56
Saline "Controls"				5	10	6 29 ± 0 33

Pooled prednisolone treated groups (1–4) Mean hemolysin titer ± S E M = 4 56 ± 0 24

Difference between group 5 and group 1 4 = 1 73 ± 0 50 (P < 0 01)

* Prednisolone solution and saline were given as intramuscular injections every second hour for the following periods a) Day —1 from 24 hours to 6 hours before the antigen injection and b) Day 0 from the time of antigen injection to 14 hours later

§ All of the mice were immunized by intraperitoneal injections of a 2 per cent suspension of sheep erythrocytes Antibody titers were measured on sera from day +5

All animals were immunized by a single intraperitoneal injection of a 2 per cent suspension of sheep erythrocytes at hour 0 They were bled on day +5

Statistical analysis The groups treated with prednisolone were subjected to analysis of variance with two criteria of classification (dose level and length of treatment) As no heterogeneity was found these groups were pooled into one prednisolone treated group (1–4)

Results Prednisolone (3 or 10 mg per day) *significantly* reduced the hemolysin titers on day +5 (comparison of group 1–4 and group 5 Table 1) Among the prednisolone treated groups 10 mg per day was not significantly more effective than 3 mg per day Supplementary administration of the hormone on day 0 did not increase the effect obtained by treatment on day 1 There was no significant interaction between dose level and duration of treatment

Experiment 2 Variation of the Duration of Prednisolone Treatment (1 mg every 2 Hours) and of the Day of its Administration (Day 1 and Day 0) Tables 2 and 3

In experiment 1 administration of prednisolone in a dose of 0 3 or 1 mg every second hour from hour —24 to hour —6 significantly reduced the hemolysin response In experiment 2 the dose of the in-

TECHNIC

Animals White male mice weighing between 17 and 28 g were randomly allotted to experimental groups by dicing Four or five days after the antigen injection all mice were bled to death by severing the jugular veins and individual sera were collected

Antigen Fresh sheep erythrocytes were washed three times with saline and diluted with saline to the concentration used To enable a post experimental control that the antigen had been deposited intraperitoneally, the antigen suspension was mixed with a small amount of Indian ink In the first three experiments by a commercial preparation ('Eyghetstusch') in the proportion 1/400 and in the last by a shellac free preparation produced for experimental purposes (Pelikan C 11/1431a (*Benacerraf et al* 1954) in the proportion 1/1300) The mice were immunized by a single intra peritoneal injection of 0 5 ml of the antigen suspension

Denomination of days and hours The day of antigen injection is called 'day 0 days before and days after conventionally indicated by minus and plus respectively Hours before and after the hour of antigen injection are similarly marked.

Titration of hemolysin Inactivated sera were titrated in 2 fold serial dilutions Hemolysin titers were determined by a standard procedure (*Berglund* 1956) However in order to reduce the amount of serum required the titrations were performed with half volumes—making a total reaction volume of 0 625 ml The degree of hemolysis was estimated with the aid of a hemolytic scale The 50 per cent end point was calculated by interpolation (*Berglund* 1956) The titers are expressed as the negative ^{2}logarithms of the serum dilutions taking 1 6.25 as a provisional zero point (1 12 5 = 1 1 25 = 2 etc) All sera from one bleeding were titrated simultaneously

Hormone treatment Prednisolone disodium 21 phosphate[1] was dissolved in sterile saline to the concentration used The corticosteroid solution was administered intra muscularly in a volume of 0 2 ml—when not otherwise stated—with intervals of one or two hours (see individual experiments)

Statistical methods The Student's t-test was used to compare the mean titers of two groups (*Fisher* 1948) Analysis of variance and calculations of regression were performed according to directions given by *Snedecor* (1946)

EXPERIMENTAL

Experiment 1 Variation of the Dose of Prednisolone (3 and 10 mg per Day) and of the Time of its Administration (Day —1 and day —1 + day 0) Table 1

Fifty mice were allotted to five groups of ten[2] Groups 1–4 received prednisolone solution These groups represented two levels of steroid dose and two lengths of treatment The treatment schedule is shown in Table 1 Group 1 was given 0 3 mg of prednisolone every second hour from hour —24 to hour 6 day —1 (a period of 18 hours), *i e* ten injections and a total dose of 3 mg Group 2 received prednisolone as group 1 on day —1, but in addition was given 0 375 mg of the hormone every second hour from hour 0 to hour +14 day 0 (a period of 14 hours)[3], *i e* eight injections and a total dose of 3 mg on day 0

Groups 3 and 4 were given prednisolone according to the time schedules of groups 1 and 2 respectively, but here each injection contained

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[1] Thanks are due to Erik Lindblom & Co (Merck Sharp and Dohme) for generous gifts of this hormone

[2] One animal from each of groups 2 and 4 were discarded on day 0 because of imperfect antigen injection

[3] On day 0 the volume of the corticosteroid solution injected was 0 25 ml

Statistical analysis Groups 1–3 and group 5, which were given injections on day —1, were subjected to an onesided analysis of variance It was found that all groups *probably* did not belong to the same population ($P<0.05$) When the control group was omitted, the remaining groups (1–3) were not significantly heterogeneous in analysis of variance Therefore, groups 1–3 could be pooled into one group (Table 2) and compared with the other groups (Table 3)

Results Administration of prednisolone solution (1 mg every second hour) on day —1 for a minimum of 6 hours—the last injection 6 hours before the antigen injection—*significantly* reduced the hemolysin level on day +4 (comparison of group 1–3 and group 5, Table 3) Treatment for 12 or 18 hours did not significantly increase the effect obtained by administration for 6 hours

On the other hand, treatment with prednisolone solution—by the same mode of administration—on day 0 for 18 hours, beginning at the time of antigen injection, had no significant effect (comparison between group 4 and group 5, Table 3) In agreement with these findings prednisolone treatment resulted in *significantly* lower antibody titers when given on day —1 than when it was administered on day 0 (comparison of group 1 3 and group 4, Table 3)

Experiment 3 Estimation of the Minimal Effective Duration of Prednisolone Administration (1 mg every Hour) on Day —1 Tables 4 and 5

In this experiment prednisolone administration on day —1 for shorter periods than used in the preceding experiments, was tested Furthermore, the prednisolone injections were given with shorter intervals

TABLE 4

Exper 3 Effects of four Different Periods of Prednisolone Administration – 12 6 4 and 2 Hours of day —1 on the Hemolysin Response *

Group no	Treatment § Kind	Treatment § Hours of administr	No of mice	Hemolysin titer Mean ± S E M
1	Prednisolone	—18→—6	9	3 96 ± 0 26
2	Prednisolone	—12→—6	8	4 71 ± 0 33
3	Prednisolone	—10→—6	10	3 56 ± 0 48
4	Prednisolone	—8→—6	10	5.32 ± 0 42
5	Saline controls"	—18→—6	10	5 57 ± 0 24

Mean hem lysin titer ± S E M (...
All of the mice were immun ...
2 per cent suspension of sheep ery ...
from day +4
§ Prednis l ne (1 mg per injecti n) and saline were given intramuscularly every h ur

dividual injections was kept constant (1 mg), but the length of treatment on day —1 was varied. Furthermore, the effect of prednisolone treatment given exclusively on day 0 was investigated (see Table 2).

TABLE 2

Exper. 2. Effects of a) three Different Lengths of Prednisolone Administration (18 Hours, 12 Hours and 6 Hours) on day —1, and b) Its Administration for 18 Hours on Day 0*, on the Hemolysin Response §*

Group no	Treatment: Kind	Day	Hours of administr.	No. of mice	Hemolysin titer Mean ± S.E.M.
1	Prednisolone	—1	—24→— 6	9	3.43 ± 0.58
2	Prednisolone	—1	—18→— 6	10	3.88 ± 0.66
3	Prednisolone	—1	—12→— 6	10	4.33 ± 0.74
4	Prednisolone	0	0→+18	10	5.86 ± 0.32
5	Saline "Controls"	—1	—24→— 6	10	6.08 ± 0.39

Mean hemolysin titer ± S.E.M. of the pooled groups (1–3) = 3.90 ± 0.38.
* Prednisolone solution was given as intramuscular injections of 1 mg every second hour. Saline was injected with the same intervals.
§ All of the mice were immunized at hour 0 by intraperitoneal injections of a 2 per cent suspension of sheep erythrocytes. Antibody titers were measured on sera from day +4.

TABLE 3

Difference (D) between Mean Hemolysin Titers of Some of the Groups of Table 2

Groups compared	Hemolysin titer Diff. ± S.E.D.	P
(1–3) and 5	2.18 ± 0.69	< 0.01
4 and 5	0.22 ± 0.51	
(1–3) and 4	1.96 ± 0.68	< 0.01

Fifty mice were allotted to five groups of ten[4]. Groups 1–4 were given 1 mg of prednisolone in solution intramuscularly every second hour for the following periods: Group 1 from hour —24 to hour —6, group 2 from hour —18 to hour —6, group 3 from hour —12 to hour —6 and group 4 from hour 0 to hour +18.

Group 5 received intramuscular injections of 0.2 ml of saline following the time schedule of group 1, and served as control.

All animals were immunized by a single intraperitoneal injection of 2 per cent suspension of sheep erythrocytes at hour 0. They were bled on day +4. The group means of hemolysin titers are found in Table 2.

[4] One animal from group 1 was later excluded from the experiment as no carbon was found intraperitoneally or elsewhere indicating that the antigen had been injected into the gut.

solone solution for four hours was sufficient to suppress the hemolysin response if a dose of 1 mg was given every hour from hour —10 to hour —6. However, it was not known whether this four hour treatment could be started at other hours on day —1 without loss of effect.

A preliminary experiment indicated that this four hour treatment was still effective when started at hour —6 but ineffective when started at hour —3. In another experiment no significant effect was obtained when this treatment was initiated at hour —24.

It was now decided to make an attempt to estimate the time limits of the prednisolone-sensitive period of day —1. In order to possibly increase the sensitivity of antibody formation to prednisolone a smaller antigen dose was given in this experiment (cf. the greater effectiveness of cortisone in rats at smaller antigen doses, *Berglund* 1956b).

TABLE 6

Exper. 4. Effect of Prednisolone Administered for 4 Hours on the Hemolysin Response. Variation of the Hour of Initiation of the Hormone Therapy.*

Group no.	Treatment §		No. of mice	Hemolysin titer Mean ± S.E.M.
	Kind	Hour of initiation		
1	Prednisolone	—24	4	4.45 ± 0.45
2	Prednisolone	—22	4	3.50 ± 1.52
3	Prednisolone	—20	4	5.70 ± 0.78
4	Prednisolone	—18	4	2.63 ± 1.27
5	Prednisolone	—16	4	5.00 ± 0.50
6	Prednisolone	—14	4	3.38 ± 0.99
7	Prednisolone	—12	4	1.50 ± 0.77
8	Prednisolone	—10	4	1.38 ± 1.06
9	Prednisolone	—8	4	2.20 ± 1.28
10	Prednisolone	—6	4	2.83 ± 1.13
11	Prednisolone	—4	4	1.75 ± 1.13
12	Prednisolone	—2	4	4.33 ± 0.73
13	Prednisolone	0	4	5.65 ± 0.67
14	Prednisolone	+2	4	4.28 ± 0.70
15	Prednisolone	+4	4	3.15 ± 1.60
16	Saline controls	10	9†	4.39 ± 0.85

* All of the mice were immunized at hour 0 by intraperitoneal injections of a 0.8 per cent suspension of sheep erythrocytes. Antibody titers were measured on sera from day +5.

§ Prednisolone (1 mg per injection) and saline were given intramuscularly every hour.

† One serum was lost during centrifugation.

Experiment 5. Estimation of the Limits of the Prednisolone-sensitive Period of Day —1 (Tables 6–8, Figures 1 and 2).

Seventy mice weighing 20–24 g were divided into four groups according to weight and randomly allotted to fifteen groups of four animals and to one group of ten. The last group received intramuscular injections of 0.2 ml saline every hour from hour —10 to hour —6,

TABLE 5

Difference (D) between Mean Hemolysin Titers of Some of the Groups of Table 4

Groups compared	Hemolysin titer Diff ± S.E.D.	P
(1-3) and 5	1.54 ± 0.41	< 0.001
4 and 5	0.25 ± 0.49	
(1-3) and 4	1.29 ± 0.46	< 0.01

Fifty rats were allotted to five groups of ten[5]. Groups 1–4 were given 1 mg of prednisolone in solution intramuscularly every hour for the following periods: Group 1 from hour —18 to hour —6, group 2 from hour —12 to hour —6, group 3 from hour —10 to hour —6, and group 4 from hour —8 to hour —6.

Group 5 received 0.2 ml of saline every hour from hour —18 to hour 6, and served as control.

All of the animals were immunized by a single intraperitoneal injection of a 2 per cent suspension of sheep erythrocytes. They were bled on day +4. Group means of hemolysin titers are found in Table 4.

Statistical analysis. All groups were subjected to an one-sided analysis of variance, which indicated heterogeneity between groups ($P<0.01$). When the control group (group 5) was omitted, it was still probable that the remaining groups did not belong to the same population ($P<0.05$). It now seemed logical to omit the group which had received the shortest treatment with prednisolone (group 4). The remaining three groups showed no significant heterogeneity and could be pooled (group 1-3, Table 4) and compared with the other groups (Table 5).

Results. As in experiment 2 the hemolysin response of mice treated with prednisolone solution for 6 hours (from hour —12 to hour —6) did not significantly differ from responses of animals, receiving the hormone for 12 hours (hour —18 to hour —6). Further, reduction of the treatment period to 4 hours (hour —10 to hour —6) did not significantly alter the level of antibody response. Thus, mice given prednisolone for a minimum of 4 hours had *significantly* lower haemolysin titers on day +4 than the controls (comparison of group 1-3 and group 5, Table 5).

On the other hand, mice treated for 2 hours (hour —8 to hour —6) produced more antibodies than animals treated for a longer period (comparison of group 1–3 and group 4, Table 5), and did not significantly differ from the control group (comparison of group 4 and group 5, Table 5).

From experiment 3 it was evident that administration of predni-

5 One animal from group 1 and two from group 2 were later excluded from the experiment because of imperfect antigen injection (cf. footnote 4).

solone solution for four hours was sufficient to suppress the hemolysin response if a dose of 1 mg was given every hour from hour —10 to hour —6. However, it was not known whether this four-hour treatment could be started at other hours on day —1 without loss of effect.

A preliminary experiment indicated that this four hour treatment was still effective when started at hour —6, but ineffective when started at hour —3. In another experiment no significant effect was obtained when this treatment was initiated at hour —24.

It was now decided to make an attempt to estimate the time limits of the prednisolone-sensitive period of day —1. In order to possibly increase the sensitivity of antibody formation to prednisolone a smaller antigen dose was given in this experiment (cf. the greater effectiveness of cortisone in rats at smaller antigen doses, *Berglund* 1956b).

TABLE 6

Exper. 4. Effect of Prednisolone Administered for 4 Hours on the Hemolysin Response. Variation of the Hour of Initiation of the Hormone Therapy*

Group no	Treatment §		No. of mice	Hemolysin titer Mean ± S.E.M.
	Kind	Hour of initiation		
1	Prednisolone	—24	4	4.45 ± 0.45
2	Prednisolone	—22	4	3.50 ± 1.52
3	Prednisolone	—20	4	5.70 ± 0.78
4	Prednisolone	—18	4	2.63 ± 1.27
5	Prednisolone	—16	4	5.00 ± 0.50
6	Prednisolone	—14	4	3.38 ± 0.99
7	Prednisolone	—12	4	1.50 ± 0.77
8	Prednisolone	—10	4	1.38 ± 1.06
9	Prednisolone	—8	4	2.20 ± 1.28
10	Prednisolone	—6	4	2.83 ± 1.13
11	Prednisolone	—4	4	1.75 ± 1.13
12	Prednisolone	—2	4	4.33 ± 0.73
13	Prednisolone	0	4	5.65 ± 0.67
14	Prednisolone	+2	4	4.28 ± 0.70
15	Prednisolone	+4	4	3.15 ± 1.60
16	Saline controls	10	9†	4.39 ± 0.85

* All of the mice were immunized at hour 0 by intraperitoneal injections of a 0.8 per cent suspension of sheep erythrocytes. Antibody titers were measured on sera from day +5.

§ Prednisolone (1 mg per injection) and saline were given intramuscularly every hour.

† One serum was lost during centrifugation.

Experiment 4. Estimation of the Limits of the Prednisolone sensitive Period of Day —1. (Tables 6–8, Figures 1 and 2)

Seventy mice weighing 20–24 g were divided into four groups according to weight and randomly allotted to fifteen groups of four animals and to one group of ten. The last group received intramuscular injections of 0.2 ml saline every hour from hour —10 to hour —6,

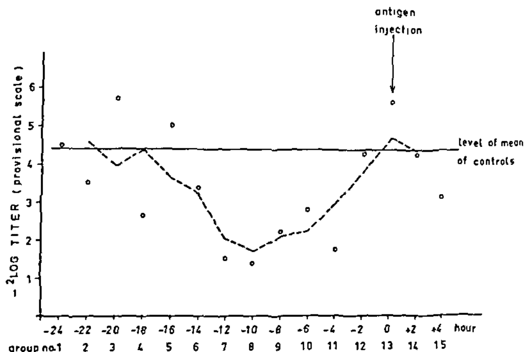


Fig 1

Exper 4 Effect of prednisolone administered for 4 hours on the hemolysin response Variation of the hour of initiation of the hormone therapy (see Table 6) Each circle represents the mean hemolysin titer (day +5) of a group and the hour when this group received its first hormone injection The horizontal line indicates the level of the mean haemolysin response (day +5) in the control group The curve has been obtained by connecting all averages of three consecutive group means from group 1 to group 15 (smoothed curve)

and served as control (group 16) The other groups were given intra muscular injections of 1 mg of prednisolone solution every hour for a period of four hours (5 injections) The beginning of the treatment period was varied among the groups as follows Group 1 received the first injection at hour —24, group 2 at hour —22, group 3 at hour —20 and so on with two hours intervals Thus group 15 was given the first injection at hour +4 All injections of prednisolone as well as of antigen were given within 2 minutes of the scheduled time

All of the animals were immunized by a single intraperitoneal injection of 0 8 *per cent suspension of sheep erythrocytes* They were bled on day +5 The group means of hemolysin titers are found in Table 6 and are plotted against the hour of the first prednisolone injection in the respective groups in Figure 1

Statistical analysis The data of the prednisolone treated groups have been subjected to regression analysis The smoothed curve in Figure 1 indicates that the group means of hemolysin titers deviate from linearity A regression analysis of haemolysin titers on the hour of the first hormone injection showed a *significant* deviation from linearity ($P<0$ 05)

If the lowest value in the curve of Figure 1 is assumed to represent

the maximum effect of the four hour prednisolone treatment used, the curve could be divided into two parts—one expressing an increasing effect (groups 1–8) and the other a decreasing effect (groups 8–15). The regression lines of these two parts have been calculated (Tables 7, 8, Fig. 2). Both lines have a significant slope and do not significantly deviate from linearity. In Figure 2 the lower 95 per cent confidence limits of both regression lines are also shown. The regression lines found apparently constitute approximations as previous experiments indicate that the curve assumes a more or less horizontal course in the beginning and the end of the period studied (cf. Figure 1).

TABLE 7

Exper. 4. Analysis of Variance including Regression for the Data of Groups 1–8 of Table 6. Regression Equation y = 3.44–0.430 (x – 5.5)

Source of variation	Degrees of freedom	Mean square	F	P
Linear regression	1	31.07	8.07	< 0.01
Deviations from regression	6	6.33	1.64	< 0.2
Error	24	3.85		

TABLE 8

Exper. 4. Analysis of Variance including Regression for the Data of Groups 8–15 of Table 6. Regression Equation y = 3.19 + 0.403 (x – 12.5)

Source of variation	Degrees of freedom	Mean square	F	P
Linear regression	1	27.28	5.85	< 0.05
Deviations from regression	6	5.45	1.17	> 0.2
Error	24	4.66		

For the purpose of this experiment another question has to be answered. How much must a group mean deviated from the mean of the control group in order to signify a statistical difference between the groups? This has been calculated from the intra group variance of the present experiment for a number of ten animals in each group and the 5 per cent level of significance. The deviation required was found to be 1.93 and is marked by a dotted line in Figure 2. The intersections between this line and the lower 95 per cent confidence limits of the regression lines constitute approximations of the limits outside which the four hour prednisolone treatment should probably not be initiated if a significant reduction of the early hemolysin response is desired.

Results. The deviation from linearity in the regression analysis of the prednisolone treated groups confirms that there is a short period of day 1 during which prednisolone can suppress the subsequent hemolysin formation. The great individual variation in hemolysin

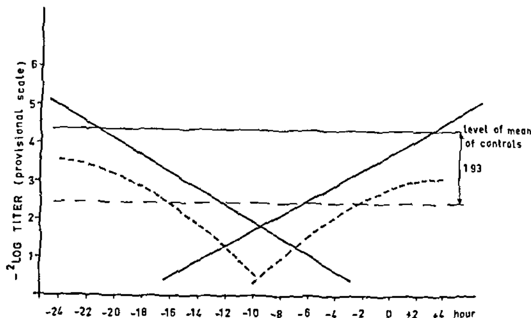


Fig 2

Exper 4 Regression lines of hemolysin titer on hour of initiation of prednisolone administration for groups 1–8 and groups 8–15 (see Figure 1 and Table 7 and 8) The dotted curves indicate the lower 95 per cent confidence limits of the regression lines

response leads to considerable spreading of the means in groups consisting of only four animals This makes calculation of the limits of this period difficult A rough approximation may, however, be obtained with the aid of the regression lines calculated and reproduced in Fig 2 As explained above this can be expressed as the time limits, outside which the four-hour prednisolone treatment used can not be expected to give statistically significant reduction of the mean hemolysin response in a specified number of animals

From the data of experiment 4 it was calculated that—if treated and nontreated groups consist of ten animals each—the four hour prednisolone treatment probably has to be started later than 16 hours after and earlier than 3 hours before antigen injection to produce a significant effect

COMMENTS

Earlier studies have shown that crystalline prednisolone and prednisone can inhibit antibody production in mice (*Foley et al 1957*) The present investigation demonstrates that prednisolone, injected every second hour as a solution, can significantly suppress the early hemolysin response in this animal Although 0 3 mg prednisolone per injection seemed to be about as effective as 1 0 mg, the larger dose was used throughout experiments 2–4 in order to take advantage of even a small increase in antibody reducing power

The observation that prednisolone was effective, when administered

before the day of antigen injection, but not when the treatment was initiated on this day, is in agreement with experiences in rats given crystalline cortisone acetate (*Berglund* 1956a) When cortisone pretreatment was supplemented by hormone administration on the day of antigen injection, the effect was significantly enhanced (*Berglund* 1956a) As shown in experiment 1 this phenomenon could not be demonstrated with prednisolone solution in mice This disagreement may be related to the difference in species, in type of hormone, in method of its administration and in dose of antigen (*Berglund* 1956b) Clarification of this question requires a careful experimental study

The studies with cortisone in rats indicated that treatment on day —1 was a requisite for an effect on the early haemolysin response (*Berglund* 1956a) The use of the minimal effective dose as well as the slow absorption of cortisone acetate—mentioned in the opening paragraph—did not permit a more accurate estimation of the length of the cortisone sensitive pre treatment period — The primary information sought by the present investigation was the limits, within which the shortest effective prednisolone treatment must be initiated, if an effect on the early hemolysin response is desired The period, thus defined, will hereafter be denoted "the prednisolone sensitive period" — In the main experiments (3 and 4), conducted to illuminate this question, prednisolone was given every hour in order to obtain a possible intensification of the effect

As a first step the effect of different lengths of prednisolone administration was investigated It was found that treatment with prednisolone solution (1 mg every hour) could be limited to 4 hours (experiment 3) In fact prolongation of this period up to 12 hours did not produce a further decrease in the hemolysin response In experiment 2 treatment for 18 hours did not significantly augment the effect obtained by administration of 6 hours In these two experiments the different administration periods all ended at 6 hours before the antigen injection (hour —6) It may therefore be objected that a still shorter effective period could have been found if the end of the treatment periods had also been varied It should be observed, however, that the period hour —10 to hour —6 is situated near the middle of "the prednisolone-sensitive period" found in the last experiment

As a second step the hour of initiation of a four hour period of treatment with prednisolone (5 injections of 1 mg) was extensively varied within 24 hours before and 4 hours after the antigen injection (experiment 4) This attempt to estimate the time limits of "the prednisolone-sensitive period" has an important limitation—the degree of antibody suppression required to prove an effect The great quantitative variation of the hemolysin response—within a group of mice—means that only large differences between groups can reach statistical significance Furthermore, different degrees of antibody reduction can not be detected with the present experimental technic

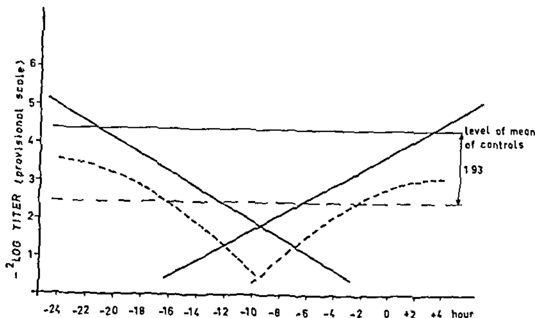


Fig 2

Exper 4 Regression lines of hemolysin titer on hour of initiation of prednisolone administration for groups 1–8 and groups 8–15 (see Figure 1 and Table 7 and 8) The dotted curves indicate the lower 95 per cent confidence limits of the regression lines

response leads to considerable spreading of the means in groups consisting of only four animals This makes calculation of the limits of this period difficult A rough approximation may, however, be obtained with the aid of the regression lines calculated and reproduced in Fig 2 As explained above this can be expressed as the time limits, outside which the four-hour prednisolone treatment used can not be expected to give statistically significant reduction of the mean hemolysin response in a specified number of animals

From the data of experiment 4 it was calculated that—if treated and nontreated groups consist of ten animals each—the four-hour prednisolone treatment probably has to be started later than 16 hours after and earlier than 3 hours before antigen injection to produce a significant effect

COMMENTS

Earlier studies have shown that crystalline prednisolone and prednisone can inhibit antibody production in mice (*Foley et al* 1957) The present investigation demonstrates that prednisolone, injected every second hour as a solution, can significantly suppress the early hemolysin response in this animal Although 0 3 mg prednisolone per injection seemed to be about as effective as 1 0 mg, the larger dose was used throughout experiments 2 4 in order to take advantage of even a small increase in antibody reducing power

The observation that prednisolone was effective, when administered

estimation of the length of this recovery period requires the elucidation of two factors 1 The length of time in which the four hour prednisolone treatment maintains an effective hormone concentration in the potential antibody forming tissue, 2 The time when the prednisolone-induced derangement of the potential antibody forming tissue begins to affect directly one or more links in the chain of events initiated by the introduction of antigen (*Berglund* 1956a)

The similarity between the effects of cortisone and total body irradiation on antibody formation has been emphasized repeatedly (Dixon 1954, *Berglund* 1956a, *Taliaferro* 1957) In a careful study using rabbits *Taliaferro & Taliaferro* (1954) found that maximal X-ray injury is evident when the animals are exposed to irradiation between 12–24 hours before the antigen injection The short, early phase of the antibody production process, which is highly sensitive to cortisone and X-ray has been called the preinduction period (*Taliaferro* 1957) In addition it has been demonstrated recently that 6-mercaptopurine is a potent inhibitor of antibody formation (*Schwartz & Dameshek* 1959) and that its action is also limited to the early phase of the antibody production process—the induction or assimilation period preceding the synthesis of antibody globulin (*Sterzl* 1960) The events which take place during this period are not fully realized This greatly hampers deliberations on the nature of the derangement caused by injurious agents (for discussion see *Taliaferro* 1957, *Wissler et al* 1957, *Berglund & Fagraeus* 1961) The discovery of the neutralizing effect which can be obtained by injections (1) of lymphoid cells, HeLa cells and yeast autolysate on the effect of X-ray (reviewed by *Taliaferro* 1957) and (2) of lymphoid cells on the effect of cortisone (*Berglund & Fagraeus* 1956 and 1961, *Fagraeus & Berglund* 1961), may open new ways to tackle this problem

Wissler et al (1957) have observed a rise in the mitotic act [illegible] stimulation by antigen or antigen modified by phagocytosis Observations in tissue culture studies support the idea that mitosis is a requisite part of the antibody production process (*La Via et al* 1960) The period of increased mitotic activity does not, however, coincide with the most radiosensitive period *Wissler et al* (1957) suggest that the most radiosensitive portion of the cellular immune reaction is located to the part of the process during which the antigen stimulus activates the reticular cell

This hypothesis involves several possible mechanisms In this publication it shall be discussed exclusively on the basis of results in experiments with corticosteroids As a result of corticosteroid therapy the phagocytic digestion of antigen may be altered to a form terminating in products, which fail to stimulate the reticular cells Several observers have found that the phagocytic digestion of antigen is delayed by cor-

The smoothed curve in Figure 1—expressing the relation between the time of initiation of prednisolone treatment and the hemolysin response—suggests that the suppression of hemolysin response gradually decreases at both ends of the period studied A more or less assymptotic approach to the level of the hemolysin response in control animals seems to be more natural than a discontinuous course of the curve If the first possibility (assymtotic course) is true, the real limits of the prednisolone-sensitive period can hardly be estimated Such a period can be measured only by finding the time limits within which prednisolone produces a defined degree of reduction of the hemolysin response As explained in the preceding paragraph the technique, used in the present experiments, only permits the estimation of two degrees of effect—significant effect and no significant effect Hence, "the prednisolone-sensitive period" is here defined by the time limits, within which initiation of prednisolone treatment produces a significant effect With a view to stressing that smaller degrees of effect can probably be produced outside this period, the period estimated will be called "the most prednisolone-sensitive period" The time limits of this period have been calculated by an approximation procedure for the case when both treated and non-treated groups consist of ten mice For the purpose of this investigation it suffices to say that "the most prednisolone-sensitive period" did not extend beyond the limits 16 hours and 3 hours before the antigen injection

It will again be stressed that these limits are expressed in terms of hour of initiation of a four-hour period of treatment with prednisolone However, a treatment started at the later time limit—hour —3— exerts its effect for at least 3 to 4 hours (cf the ineffectiveness of prednisolone administration for a two-hour-period, experiment 3) Determination of the length of this period requires knowledge of the half-life of prednisolone, given intramuscularly as a solution in mice[r] and the minimal effective plasma concentration of the hormone required to derange the potential antibody forming tissue Without this information it is not possible to estimate the latter time limit of the period, during which a prednisolone-induced damage to the potential antibody forming tissue leads to reduction of the early haemolysin response In other words, we do not know how late—in relation to the antigen injection—an effective tissue concentration of prednisolone ceases to influence the processes initiated by the introduction of the antigen From what is said above it follows that this time limit is probably not located earlier than hour 0—the time of antigen injection

The apparent ineffectiveness of the four-hour prednisolone treatment, when started 20–24 hours before the antigen injection, suggests that the potential antibody forming tissue recovers rather rapidly An

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[r] In man the half life of intravenously administered prednisolone solution has been found to be 204 241 minutes (*Nugent et al* 1959)

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Wissler et al (1957) have observed a rise in the mitotic activity in the red pulp of the rat spleen about 48 hours after injection of antigen They propose that this is evidence of a division of reticular cells, which proliferate after stimulation by antigen or antigen modified by phagocytosis Observations in tissue culture studies support the idea that mitosis is a requisite part of the antibody production process (*La Via et al* 1960) The period of increased mitotic activity does not, however, coincide with the most radiosensitive period *Wissler et al* (1957) suggest that the most radiosensitive portion of the cellular immune reaction is located to the part of the process during which the antigen stimulus activates the reticular cell

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tisone (*Lurie et al* 1952, *Kass et al* 1953, *Clawson & Nerenberg* 1953) No studies of qualitative effects have been reported Indirect evidence was provided by studies on rats with cortisone and a primary antigenic stimulation with S *typhi* H Treatment with cortisone (4 mg/100 g per day) for 9 days (day —6 through day +2) resulted in a significantly lower agglutinin titer on day +6 This effect was still present on day +18 and day +27—a period of rising antibody level in the nontreated animals In fact the difference seemed to be greatest on day + 27 It is probable that intensive antibody formation during such a long period requires continuous stimulation by persisting antigen Delayed phagocytic digestion of antigen can hardly be manifest for such a long period after the end of cortisone administration One possible explanation might be that the digestion of antigen in the early phase is qualitatively defective and that the number of effective antigen-units thereby is definitely diminished

Another possible mechanism would be that corticosteroids make the reticulum cell refractory to the stimulus of antigen A similar cellular state of non-reactivity has been assumed to be the cause of repressed inflammatory reaction during cortisone administration (review in *Menkin* 1960) Leukotaxine and leukocytosis-promoting factors are produced in less than normal amounts by cells exposed to the inflammation-inducing agent

The refractority of the reticulum cells may be manifest as a failure to divide when stimulated by antigen *Menkin* (1953) observed that cortisone and hydrocortisone suppressed the normal cleavage of fertilized sea urchin ova

An inability of the reticulum cell to divide can be caused by direct inhibition of mitosis That cortisone can inhibit mitosis is well substantiated (review in *Ghadially & Green* 1957) *Craig* (1952) studied the histology of antigenically stimulated lymph nodes in rabbits given cortisone He found mitotic inhibition in the cortex and secondary germinal centers It has also been shown that hydrocortisone reduces the incorporation of P^{32} into nucleic acids of normal lymphatic cells *in vitro* (*Kit et al* 1954)

A further indication that cortisone impairs proliferation of reticuloendothelial cells emanates from studies of phagocytosis When a large dose of cortisone was administered to rats given an intravenous blocking dose of carbon, regeneration of the bloodclearing phagocytotic function was inhibited (*Benacerraf et al* 1954)

It is obvious that nothing but further extensive experimentation—especially at the cellular level—can clarify the mechanism by which corticosteroids inhibit antibody formation A definite concept will probably not be accessible until the induction or assimilation phase of antibody production is more fully realized It is a reasonable prediction that studies with inhibitory substances also may serve to clarify the normal events during this phase

SUMMARY

A study has been made of certain time factors, which condition the suppressing effect of prednisolone on antibody formation in mice The test object has been the early hemolysin response after a single intraperitoneal injection of sheep erythrocytes Prednisolone was administered hourly or every second hour as intramuscular injections of a saline solution of prednisolone disodium 21-phosphate

1 Administration of a large dose of prednisolone during a four hour period—from 10 hours to 6 hours before the antigen injection—produced a significant effect

2 Prolongation of this treatment period did not appear to increase the effect

3 Administration of prednisolone from the hour of antigen injection to a) 18 hours later had no significant effect in itself and b) 14 hours later did not increase the reducing effect obtained by treatment on day —1

4 By an approximation method it was estimated that the four-hour treatment with *prednisolone should be initiated later than 16 hours and* earlier than 3 hours before the antigen injection if a statistically significant effect were desired in an ordinarily sized experiment (ten animals both in the prednisolone treated and in the control groups)

The limited significance of "the most prednisolone sensitive period", thus defined, is commented on Possible mechanisms by which corticosteroids may inhibit the antibody production process, are discussed

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BACTERIAL GROWTH IN URINE

By

GÖRAN AURELIUS

Received 29 xi 61

In recent years several authors (*Kass* 1956, *Sanford et al* 1956) have pointed out the importance of carrying out not only qualitative but also quantitative bacteriological studies in cases of urinary tract infections, especially chronic pyelonephritis From the number of bacteria occurring in the specimen it is possible to estimate whether these play an etiologic rôle or are to be looked upon only as contamination Infection is considered to exist when the number of bacteria exceeds 100 000 per ml

To achieve an exact measurement of the number of bacteria it is necessary to perform the bacteriological study directly after the specimen is obtained This is possible only in hospitals equipped with bacteriological laboratories Otherwise, transportation time together with the temperature and acidity prevailing in the specimen during transit must be taken into consideration

With a view to this it has been considered of value to form a picture of the velocity with which the most common pathogenic bacteria of the urinary tract grow in urine at different temperatures and pH

The following bacteria were examinated Streptococcus faecalis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli and Proteus mirabilis The strains were obtained from fresh routine specimens

METHODS

A medium portion of urine sampled from a healthy man in which albumin glucose and formed pathological elements could not be demonstrated was used as substrate Only sterile urine was employed in the exper[illegible]



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I Broth

II Urine pH 5 1

III Urine pH 6 2

IV Urine pH 7 4

24 hours

a STREPTOCOCCUS FAECALIS

I Broth

II Urine pH 5 3

III Urine pH 6 5

IV Urine pH 7 5

24 hours

b STAPHYLOCOCCUS AUREUS

Fig 1

technique Two plates were poured from each dilution and the average number of bacteria calculated Experiments were made at three different pH ranges 5 0–5 3, 5 7–6 5 and 7 3 7 6 After 24 hours pH was reestimated For comparison nutrient broth (pH 7 4) was incubated with the same number of bacteria, the growth was studied at the same time intervals as for the urine specimens pH was calculated by a potentiometer

The bacterial growth is expressed in 2-logarithms to give a direct measure (Monod 1949) of the number of doublings and halvings of the original bacterial number

RESULTS

The growth of Streptococcus faecalis (Fig 1a) in urine at pH 6 2 and 7 4 was very similar to growth in broth, while the growth at pH 5 1 seemed to be completely arrested Under all conditions no growth was demonstrable at +4° C No change in pH could be detected after 24 hours

Similar results were found for Staphylococcus aureus (Fig 1b) except that growth at pH 5 3 and +37° C was fair here

The growth of Escherichia coli and Proteus mirabilis (Fig 2a and 2b) was very different from growth of the other bacteria examined At pH 5 0 the initial number of bacteria would decrease after 24 hours, the decrease being most pronounced at +37° C The urine, however, did not reach sterility Even at the higher pH-values at +4° C a decrease in the bacterial number was demonstrable, but at higher temperature the growth was satisfactory At +20° C and +37° C in the pH 7 5–7 6 samples a reduction to pH 7 2–7 3 was seen after 24 hours No changes of the initial pH-values were noticed

The growth of Pseudomonas aeruginosa (Fig 3) in urine at pH 6 2 and 7 3 was as good as in broth—in fact at pH 7 3 a four-fold increase in the initial number of bacteria was manifest even at +4° C Growth was demonstrable also at pH 5 0 and +37° C An increase in pH to 7 4–7 6 was noticed at the 7 3 value after 24 hours, but no change was found at other values

DISCUSSION

As judged from the limited number of bacterial strains tested the investigation shows that refrigeration of the urine specimens to +4° C will guarantee a constant bacterial number for at least 6 hours If specimens are kept at room temperature, no more than 4 hours must pass before cultivation is performed Otherwise the risk of a considerable change in the bacterial number may be involved Also the number of bacteria in a urine specimen may vary considerably accord

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Fig 1

The growth of Streptococcus faecalis and Staphylococcus aureus in broth and urine

N_t = the bacterial number per 0 5 ml urine (broth) at the time t

N_0 = the initial bacterial number

———— = +37° C ...... = +22° C - - - = +4° C

$\log_2 x^t\ x^0$ I Broth 20 15 10 5 0 2 4 6 24 hours

$\log_2 x^t\ x^0$ II Urine pH 5 1 20 15 10 5 0 2 4 6 24 hours

$\log_2 x^t\ x^0$ III Ur ne pH 6 2 20 15 10 5 0 2 4 6 24 hours

$\log_2 x^t\ x^0$ IV Urine pH 7 4 20 15 10 5 0 2 4 6 24 hours

a STREPTOCOCCUS FAECALIS

$\log_2 x^t\ x^0$ I Broth 20 15 10 5 0 2 4 6 24 hours

$\log_2 x^t\ x^0$ II Urine pH 5 3 20 15 10 5 0 2 4 6 24 hours

$\log_2 x^t\ x^0$ III Ur ne pH 6 5 20 15 10 5 0 2 4 6 24 hours

$\log_2 x^t\ x^0$ IV Urine pH 7 5 20 15 10 5 0 2 4 6 24 hours

b STAPHYLOCOCCUS AUREUS

Fig 1

$\log_2 x^t - x^0$ I Broth

20 15 10 5 0

2 4 6 24 hours

$\log_2 x^t\ x^0$ II Urine pH 5 0

0 -5 10 15 20

2 4 6 24 hours

$\log_2 x^t\ x^0$ III Urine pH 5 4

20 15 10 5 0

2 4 6 24 hours

$\log_2 x^t\ x^0$ IV Urine pH 7 6

20 15 10 5 0

2 4 6 24 hours

a ESCHERICHIA COLI

$\log_2 x^t\ x^0$ I Broth

20 15 10 5 0

2 4 6 24 hours

$\log_2 x^t\ x^0$ II Urine pH 5 0

0 5 10 15 20

2 4 6 24 hou s

$\log_2 x^t\ x^0$ III Urine pH 5 7

20 15 10 5 0

2 4 6 24 hours

$\log_2 x^t\ x^0$ IV Urine pH 7 5

20 15 10 5 0

2 4 6 24 hou s

b PROTEUS MIRABILIS

Fig 2

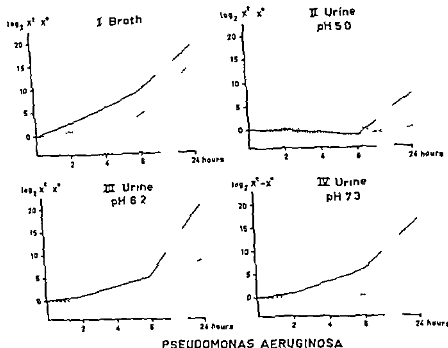


Fig 3

The growth of Pseudomonas aeruginosa in broth and urine
X_t — the bacterial number per 0.5 ml urine (broth) at the time t
X_o — the initial bacterial number
——— = + 37° C. ——— = + 22° C - - - - - - - = + 4° C.

ing to the length of time the urine has been retained in the bladder If one urine specimen is taken 2 hours after micturition and another 6 hours after micturition, it is very likely that the number of bacteria will be different on the latter occasion A standardization of conditions for the taking of specimens is therefore desiderable

As pH of the urine can vary from day to day, the bacterial content in the urine can fluctuate according to this Therefore a control of the pH of the urine specimen is to be recommended, so that the result of the bacteriological study can be evaluated against the background of the pH value

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Fig 2

The growth of Escherichia coli and Proteus mirabilis in broth and urine
X_t — the bacterial number per 0.5 ml urine (broth) at the time t
X_o — the initial bacterial number
——— = + 37° C. ——— = + 22° C. - - - - = + 4° C.

I Broth
$\log_2 x^t/x^0$
20
15
10
5
0
2 4 6 24 hours

II Urine pH 5 0
$\log_2 x^t/x^0$
0
-5
10
15
20
2 4 6 24 hours

III Urine pH 6 4
$\log_2 x^t/x^0$
20
15
10
5
0
2 4 6 24 hours

IV Urine pH 7 6
$\log_2 x^t/x^0$
20
15
10
5
0
2 4 6 24 hours

a ESCHERICHIA COLI

I Broth
$\log_2 x^t/x^0$
20
15
10
5
0
2 4 6 24 hours

II Urine pH 5 0
$\log_2 x^t/x^0$
0
5
10
15
20
2 4 6 24 hours

III Urine pH 5 7
$\log_2 x^t/x^0$
20
15
10
5
0
2 4 6 24 hours

IV Urine pH 7 5
$\log_2 x^t/x^0$
20
15
10
5
0
2 4 6 24 hours

b PROTEUS MIRABILIS

Fig 2

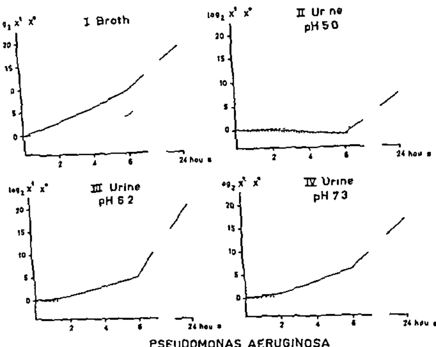


Fig 3

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Xo — the initial bacterial number
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Xt — the bacterial number per 0.5 ml urine (broth) at the time t
Xo — the initial bacterial number
———— = + 37° C, — = + 22° C, — = + 4° C

$\log_2 x^t - x^o$ I Broth

20 15 10 5 0

2 4 6 24 hours

$\log_2 x^t - x^o$ II Urine pH 5 0

0 -5 10 -15 20

2 4 6 24 hou s

$\log_2 x^t x^o$ III Urine pH 6 4

20 15 10 5 0

2 4 6 24 hours

$\log_2 x^t x^o$ IV Urine pH 7 6

20 15 10 5 0

2 4 6 24 hou s

a ESCHERICHIA COLI

$\log_2 x^t x^o$ I Broth

20 15 10 5 0

2 4 6 24 hours

$\log_2 x^t x^o$ II Urine pH 5 0

0 5 10 15 20

2 4 6 24 hou s

$\log_2 x^t x^o$ III Urine pH 5 7

20 15 10 5 0

2 4 6 24 hours

$\log_2 x^t x^o$ IV Urine pH 7 5

20 15 10 5 0

2 4 6 24 hours

b PROTEUS MIRABILIS

Fig 2

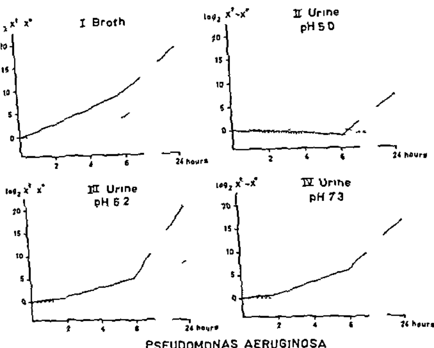


Fig 3

The growth of Pseudomonas aeruginosa in br th and urine
X_t — the bacterial number per 0 5 ml urine (broth) at the time t
X_0 — the initial bacterial number
———— + 37° C ——— + 22° C - - - - - + 4° C

ing to the length of time the urine has been retained in the bladder If one urine specimen is taken 2 hours after micturition and another 6 hours after micturition it is very likely that the number of bacteria will be different on the latter occasion A standardization of conditions for the taking of specimens is therefore desiderable

As pH of the urine can vary from day to day, the bacterial content in the urine can fluctuate according to this Therefore a control of the pH of the urine specimen is to be recommended, so that the result of the bacteriological study can be evaluated against the background of the pH value

---

Fig 2

The growth of Escherichia coli and Proteus mirabilis in broth and urine
X_t — the bacterial number per 0.5 ml urine (broth) at the time t
X_0 — the initial bacterial number
———— + 37° C ——— + 22° C - - - - - + 4° C

SUMMARY

The growth in urine of some common pathogenic bacteria of the urinary tract has been studied by means of colony count On the basis of these results suitable conditions for taking and transporting specimens of urine for bacteriological examination are discussed

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FROM THE BLOOD TRANSFUSION SERVICE, UNIVERSITY HOSPITAL, UPPSALA
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STOCKHOLM, SWEDEN

SEROLOGICAL ADHESION OF RED CELLS TO HUMAN FOETAL KIDNEY AND LUNG CELL CULTURES

I. *The Activity of Blood Group A and B Antibodies and other Thermostable Factors in Human Serum*

By

CLAES F. HÖGMAN

Received 12.xii.61

Mixed agglutination (2) is a useful method for demonstration of blood group antigens on the surface of cells other than erythrocytes. However, in a previous publication (15) it was shown that reactions sometimes occurred which deviated from the expected reaction pattern. Some of these reactions seemed to be associated in some way with A and B blood group antigens and antibodies. Consequently, the occurrence of atypical reactions must be due to a mechanism other than the one of specific mixed agglutination.

Under the names of the adhesion phenomenon (6, 28), serological adhesion (19, 20), and immune adherence (22), adsorption of extraneous microscopic particles to particulate antigens in the presence of antibody and complement has been described. Taken in the widest sense the first observation of this kind seems to have been made by *Levaditi* in 1901 (21), who observed that *Vibrio cholerae*, injected into immunized guinea pigs or rabbits became coated by blood platelets. This phenomenon also occurred if bacteria sensitized *in vitro* by exposure to immune serum were injected into normal guinea pigs. In his review on the subject *Lamanna* (19) states that the above mentioned terms and others such as the Rieckenberg reaction, Levaditi phenomenon, Laveran-Mesnil reaction, and thromb[illegible] refer to the same [illegible] step of the reactio[illegible] next all four class[illegible] antibody complex [illegible] capable of attracting quite unrelated foreign particles such as red or white cells, thrombocytes, colloidal silica etc. (20). On the other hand *Nelson & Nelson* (23, 24) claim that this is an over-simplification. They distiguish between acid adhe

This study was aided by a grant from the *Swedish Cancer Society*. For excellent technical assistance the author is indebted to Miss *Britt Westerdahl*.

sion and immune adherence In both cases red cells are attracted to an antigen-antibody-complement complex However, the acid adhesion phenomenon only occurs at a pH below five, it is inhibited by high salt concentrations, the test red cells react independently of their species, and the adhesiveness of the red cells is not influenced by treatment with tannic acid or trypsin The immune adherence phenomenon, on the other hand, acts over a wide pH range, it occurs with human and monkey erythrocytes only, it is not inhibited by high salt concentrations, the rate of reaction is dependent on temperature, and it is completely inhibited by treatment of the indicator red cells with tannic acid or trypsin *Nelson & Nelson* (24) also found that the adherence of precipitate-particles to sheep red cells occurred only in the presence of antibody to the erythrocytes This type of reaction they named "mixed aggregation"

In most previous publications the antigens have consisted of different types of bacteria, but other particles such as starch (26, 27) have also been used As far as the present author is aware no investigations on human organ cells have been published

The observations mentioned indicate that complement or some other nonspecific serum factor, referred to below as accessory factor, may interfere in the mixed agglutination reaction as was proposed in a previous paper (15) The present study is concerned with investigations on the thermostable serum factors and the indicator particles of a homologous system consisting of human foetal kidney and lung cells, human serum and human red cells The ABO blood group system is used as a model The nature of the accessory factor and its relation to the complement system is considered in a subsequent paper (9) A schematic description of the theoretical background to the reactions is presented in Figure 1

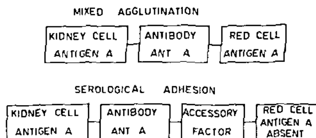


Fig 1

MATERIAL AND METHODS

Tissue cultures Trypsinized cells from human foetal kidney and lung were grown on the wall of neutral glass test tubes (13) The culture medium consisted of balanced salt solution (BSS Hanks or Earle) with the addition of 0 5 per cent
serum and five per cent human group AB
as heat inactivated and sterile filtered by
medium was changed after five days and
enance medium the serum concentration

was reduced to two per cent The cultures were used in the tests usually within two weeks Immediately before use they were washed once with phosphate buffered saline (PBS), pH 7 2-7 4

Human serum was drawn from healthy blood donors and from donors immunized with blood group A or B substance (Knickerbocker Biosales, New York) In the mixed agglutination experiments serum was inactivated for 30 minutes at 56° C or, if the controls indicated that this was not sufficient, for another ½ hour

As the source of accessory factor fresh serum was used on the day of withdrawal or after storage at —30° C or —70° C for up to one month The serum was tested with non sensitized tissue in order to ascertain that it did not spontaneously give a positive reaction and was diluted in order to get optimal reactivity, usually 1/20–1/30 (cf 9)

Mixed agglutination (abbreviated below as MA) was performed according to the method described in (15) All batches of tissue cultures were checked with this method for presence of the ABO antigen expected from the blood group of the corresponding foetus (cf 13, 14)

Serological adhesion (abbreviated below as SA) was performed according to the following procedure

1 Washing of tissue cells once with PBS

2 Sensitization of the cells by incubation with 0 2 ml amounts of inactivated serum undiluted or diluted with PBS, incubation at room temperature (20–22° C) for 60 minutes if not otherwise stated

3 Washing of the cells once with PBS and subsequent removal of liquid by

opriate dilutions of fresh human

7 6 and an ionic strength of 0 14a

5 Washing of the cells three times with PBS

6 Pipetting of 1 ml volumes of 0 5 per cent suspensions of red cells into the culture tubes

7 After incubation of the cultures in a slightly inclined position for 1 hour, during which the red cells formed a sediment on the layer of cultured cells, the tubes were rocked gently 4-5 times and placed vertically in a rack. After another hour the tubes were inspected under the microscop

Adsorption of red cells on the surface

considered as a positive reaction (see

and the evaluation of the reaction str

RESULTS

After incubation with heat-inactivated or aged serum

suitable

of group

of group

cells of

control

sion and immune adherence In both cases red cells are attracted to an antigen-antibody-complement complex However, the acid-adhesion phenomenon only occurs at a pH below five, it is inhibited by high salt concentrations, the test red cells react independently of their species and the adhesiveness of the red cells is not influenced by treatment with tannic acid or trypsin The immune adherence phenomenon, on the other hand, acts over a wide pH range, it occurs with human and monkey erythrocytes only, it is not inhibited by high salt concentrations, the rate of reaction is dependent on temperature, and it is completely inhibited by treatment of the indicator red cells with tannic acid or trypsin *Nelson & Nelson* (24) also found that the adherence of precipitate-particles to sheep red cells occurred only in the presence of antibody to the erythrocytes This type of reaction they named "mixed aggregation"

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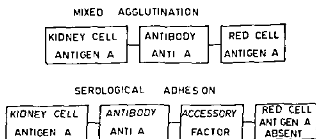


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4 Incubation with 0 2 ml amounts of appropriate dilutions of fresh human serum in PBS or ... 7 6 and an ionic strength of 0 145

suspensions of red cells into the ... tubes

RESULTS

After incubation with heat ...
suitable blood group ...
of group A and AB ...
of group A (13, 14), ... mixed agglutination (2) Red cells of group 0 serve as a negative control

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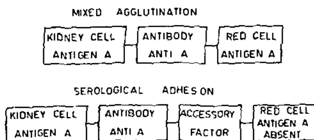


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with red cells not only of group A but also of group O The fresh serum did not contain anti-A and did not give positive reaction by itself

TABLE 2

SA Reaction of Red Cells of Group O with Lung Cells of Group A (No 12/61) First Sensitized with Inactivated anti A Serum (No 11) Non Absorbed and Absorbed with Different Samples of Red Cells and Subsequently Incubated with Fresh Group AB Serum (No 1278) Diluted 1/20

Anti A serum (no 11) Absorbed with red cells of groups	Sensitized cells	LBS control
Non absorbed	+++	—
O (RP + 13590)	+++	—
O (BJ + BL + 15017)	+++	—
O (BL + SJ + 13683)	+++	—
A_1 (1468)	—	—
A_1 (1699)	—	—
A_2 (1722)	—	—
A_2 (1774)	—	—
Buffer control	—	—

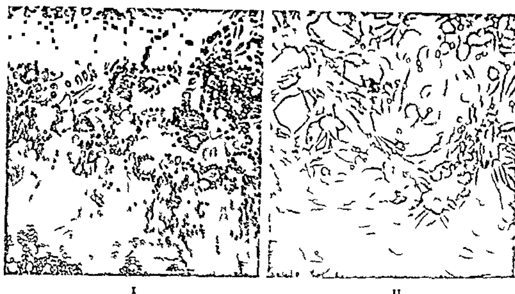
The anti A serum was tested in dilutions 1/5 and 1/10

Table 2 demonstrates an experiment in which this agglutinating-provoking thermostable factor in serum is removed by absorbtion with red cells of group A but not of group O The thermostable factor thus behaves as an antibody with the specificity anti-A Similarly, the activity was removed from a serum containing anti-B by absorption with group B red cells, when subsequently tested with tissues from group B foetuses By zone electrophoresis the activity was found in the gamma globulin region (17) This type of reaction requiring both antibody and accessory factor is referred to as serological adhesion (SA) The degree of thermo resistance of the antibody was tested by treatment of a serum containing anti A (no 11) for longer periods No measurable loss of activity was found in the MA or SA test after 8 hours' treatment at 56° C, whereas the titre was 1–2 steps lower after 16 hours' inactivation

TABLE 3

SA Reaction of Red Cells of Group O with Kidney Cells from a Group O Foetus (No 105/61) after Treatment of the Kidney Cells with Fresh Serum from 35 Blood Donors

Reciprocal of serum dilution	Number of sera				Total
	Blood group of serum donor				
	O	A	B	AB	
25	–	2	–	–	2
5	2		–	2	4
1	10	3	3	3	19
Positive	12	5	3	5	25 (70%)
Negative	3	3	–	4	10



I II

Fig 2

Microphotographs of two kidney cell cultures *from a group A foetus sensitized with* anti A (I) and non sensitized (II) Both cultures were incubated with fresh group AB serum diluted 1/20 test red cells of group O Magnification 8 × 16

TABLE 1

Reactions Obtained with Undiluted Fresh or Heat Inactivated Sera from Healthy Blood Donors when Tested with Kidney Cultures from a Foetus of Blood Group A (No 190/60)

Serum		Fresh serum		Heat inactivated serum		
		Test red cells				
no	Blood group of donors	A_1 (CH)	0 (BM)	A_1 (CH)	0 (BM)	Titre (A_1 red cells)
8261	A	—	+	—	—	
8264	A	+	++	—	—	
8265	A	++	+++	—	—	
8279	A	+++	++	—	—	
8220	B	++	++	—	—	
8231	B	++	+++	—		
8236	B	(+)	+		—	
8280	B	+++	+++		—	
8266	O	+++	+++	+++	—	4
8270	O	++++	+++	++++	—	8
8271	O	++++	+++	+++	—	4
8284	O	—	—	—	—	

If however fresh serum is used reactions of the nature shown in Table 1 can be expected indicating the participation of one or several labile serum factors In order to inactivate those factors serum was heated at 56° C for 30 minutes or in some cases for 60 minutes

Cells from group A foetuses which had been incubated first with inactivated serum containing anti A and then with fresh serum reacted

abolished the adhesiveness of the red cells in the SA reaction The MA reaction on the other hand was not affected

Red cells from the umbilical cord of three group O full term infants gave positive SA tests although the reactions were much weaker than with most adult red cells

TABLE 4

The Adhesiveness in the SA Test of Red Cells Kept at 37° C and at 4° C

Red cells	Washing of red cells at temp	Reciprocal of dilution of fresh serum						
		1	5	2	12	6?	3125	Buffer control
Y K	37°	+++	+++	++++	++	(+)	—	—
	4°	+++	+++	+++	++	(+)	—	—
3070	37°	+++	+++	++	++	—	—	—
	4°	++	+++	++	+	—	—	—
M	37°	+++	+++	+++	++	—	—	—
	4°	++	++	+++	+	—	—	—

Kidney cells of group O (No 124/61) sensitized with serum 9464

When red cells of other species are used as detector cells in the present system it has to be taken into account that some of these cells contain A like and/or B like antigens A typical MA reaction can be obtained with pig red cells of group A (16) in the system containing human kidney cells of group A and antibody of anti A

TABLE 5

MA Reactions Obtained with Lung Cells from a Group B Foetus (No 148 60) and Red Cells from Different Animals Containing B like Antigens

Test serum (K)* absorbed three times with red cells	Test erythrocytes					
	Guinea pig	Pig	Dog	Rabbit	Human B	Human O
Non absorbed	+++	++++	++++	++++	++++	—
Human group O	++	++++	++++	++++	++++	
Horse	—	++	++	+++	++++	
Guinea pig	—	—	—	++	++++	
Pig	—	—	—	—	++++	
Dog	—	—	—	—	++++	
Rabbit		—	—	—	++++	
Human group B	—	—	—	—	+++	
Saline control	—	—	—	—	—	

* The test serum was obtained from a group O person immunized with group B substance

Table 5 shows that the B like red cell antigen of some different animals (guinea pig pig dog and rabbit) may also give a positive MA reaction with certain human anti B sera This experiment demonstrates the complexity of the group B antigen of the human foetal kidney The

However, when fresh sera were used it was found that some of these caused positive reactions which could not be due to A or B antigens and antibodies Thus, certain group AB sera acted on tissue cells of any ABO blood group and certain sera, irrespective of ABO blood group, reacted with group O tissue Table 3 exemplifies this by demonstrating that 70 per cent of the sera tested, irrespective of their ABO blood group, caused positive reactions with group O kidney cells

If a positive serum was heated for 30 minutes at 56° C and then incubated with group O kidney cells no reaction occurred But after washing and further incubation of the kidney cells with a fresh negative serum the cells were able to adsorb indicator red cells Even this type of reaction thus was dependent upon one or several thermostable factors—*in this case not blood group antibodies, however*—and one or several thermolabile accessory factors The thermostability of the antibody-like factors was found to be as great as that of the anti-A described above The factors thus resisted heating at 56° C for 16 hours

The negative reactions in the experiment shown in Table 3 were demonstrated not to be due to lack of accessory factor since positive reactions in dilutions above 1/125 of the same accessory sera were obtained with kidney cells sensitized with a heat-inactivated positive serum

To interpret these results further experiments are needed It seems possible, however, that the thermostable factors are naturally occurring antibodies against organ tissue antigens of non-blood-group specificity

Investigation of the Test Red Corpuscles

Experiments on the possible influence of incomplete cold antibodies As the red cells were often kept for some time at 4° C before use, the adhesiveness might be connected with the fixation of incomplete complement-fixing cold antibodies which are normally present in human serum (5) In order to test whether such sensitization of the red cells was part of the SA mechanism three experiments of the type shown in Table 4 were performed Each time three different group O donors were bled in pre warmed glass tubes The samples were kept throughout at 37° C and washed three times with saline at this temperature Portions of the same samples were placed for two hours at 4° C and subsequently washed at room temperature The reactivity of the cells was the same in both cases when tested with SA It could be concluded that sensitization of the red cells with cold antibodies prior to the tests was not responsible for the reaction

Experiments with trypsinized human red cells, with red cells of new born infants, and with red cells from other species Mild treatment of the human red cells with trypsin, as used in blood group serological work when some incomplete antibodies are to be tested, completely

in one and the same reaction some particles will function and others will not (22) The reason why is unknown Among the red cells of different species those of primates often seem to react more readily than those of lower animals (28) But even within the same species variations in the adhesiveness of the test red cells may appear This has been shown for trypanozomes by *Brown & Broom* (1) and for human foetal organ cells by *Hogman* (15) No correlation to any disease (1), blood group (15) or to the secretor property (15) of the donor of the test red cells has hitherto been found

In the present type of reaction guinea pig and rabbit red cells did function as detector particles (although giving much weaker reactions than most adult human red cells), whereas monkey red cells did not react This does not conform with the requirements for the immune adherence (IA) reaction in which, according to *Nelson* (22), only primate red cells can be used Furthermore, the IA reaction is said to need whole complement whereas it is shown in a subsequent paper (9) that the accessory factor in the present reaction does not consist of all of the four classical complement components In some other respects, however, both types of reaction showed the same properties occurrence at neutral pH values and abolishment of the reaction by treatment of the indicator red cells with trypsin There was no indication that the adhesiveness of the red cells depended upon sensitization Incomplete cold antibodies, being the only known erythrocyte auto-antibodies which are regularly present in the human blood (5), were excluded in this respect Thus the present reaction is not identical with acid adhesion or immune aggregation

Since the name of serological adhesion (19) seems to have been used to cover reactions of related kinds even if their mechanisms show differences in details, this term has been adopted for the present reaction

Conglutination (7), in recent years extensively studied by Coombs and collaborators (for references see 4), may be defined as the clumping of red cells which have been first sensitized by an appropriate anti body, then coated by a non lytic type of complement, and finally exposed to the action of a thermostable substance, conglutinin, present in bovine serum This reaction shows some characteristics which are similar to those of serological adhesion Thus, in conglutination the complement components C 1, C'4, and C'2 take part in the reaction But in addition a thermostable serum component, conglutinin, is necessary apparently acting as an 'agglutinating factor" after the complement components have been fixed to the sensitized cells (4) The thermostable antibody like factors described in the present paper are bound to the cells before the thermolabile component can act Thus, they cannot be identical with the conglutinin Furthermore, the accessory factor in the present SA system was very potent in fresh serum (see9) Since it is stressed by *Coombs et al* (4) that the content

most probable interpretation of the experiment is that the human serum contained a mixture of antibodies with differing anti-B specificity Part of these sub-specificities correspond to the B-like antigens of the different animals which are thus capable of absorbing fractions of the antibodies However, one fraction reacts only with the human kidney and red cell blood group B antigens but not with the red cell group B antigen of the horse, guinea pig, pig, dog, and rabbit

In performing the SA test it was therefore necessary to do it two stages, the first one with inactivated antibody-containing serum, the second stage with fresh serum as the source of accessory factor A saline control (i e cells not incubated with fresh serum) was included in the tests as a means of distinguishing between MA and SA reactions With these precautions no SA reaction was obtained with red cell samples from three Cynomologous monkeys Weak reactions of the SA type were obtained with the red cells of three guinea pigs and two out of three rabbits MA occurred with sheep red cells in the A-anti-A system

DISCUSSION

Owing to technical difficulties the antigenic structure of human organ cells is much less completely mapped than that of the red cells However, by the use of fluorescent antibodies (10, 11, 25) and mixed agglutination (2, 12, 13, 14) our knowledge, especially about the ABO antigens, has increased in recent years The mixed agglutination technique has the advantage but also the limitation that only antigens present in the "detector" red cells can be investigated By combining this method with the antiglobulin technique (3) and by the use of the tanned cell technique (8) a wider antigenic spectrum can be tested In the present communication an additional phenomenon has been found to occur when tissue cells have combined with antibodies directed against the cellular surface As a model the blood group A and B antigens and antibodies have been used By further fixation of accessory factors present in fresh human serum, the sensitized cells acquire the capacity of adsorbing the detector red cells which, contrary to the requirement in mixed agglutination, lack the relevant blood group antigen

However, certain fresh human sera give rise to reactions which cannot be due to A or B antibodies (15) These reactions are caused by one thermostable component which may be a "natural" antibody against tissue cells and one thermolabile accessory factor, which seems to be the same factor as in the reaction system tissue cells of blood goup A—anti-A—accessory factor—group O red cells Further studies on the nature of these supposed antibodies are in progress

Quite different kinds of detector particles have been shown to act in different types of SA reactions (19, 24), but it is also known that

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SEROLOGICAL ADHESION OF RED CELLS TO HUMAN FOETAL KIDNEY AND LUNG CELL CULTURES

2 *The Role of Human Complement*

By

KARL ERIK FJELLSTRÖM and CLAES F HÖGMAN

Received 12.xii.61

In a preceding paper (4) a phenomenon has been described consisting of adhesion of human erythrocytes to sensitized human kidney or lung cells This adhesion differed from mixed agglutination in being elicited only in the presence of a heat labile serum factor, referred to below as accessory factor The heat labile factor was apparently non specific and was present in all normal sera tested This phenomenon showed many similarities to the so called serological adhesion (6) or immune adherence phenomena (12) The first of these two terms has been used here since it seems to cover reactions of similar but not quite identical nature

The present communication is concerned with studies on the thermolabile accessory serum factor and its relation to the complement system

MATERIAL AND METHODS

The tissue material and the technical pro[illegible] n the serological adhesion (SA)

determination of complement

[illegible] each reagent was [illegible] se to 100 per cent haemolysis together with each [illegible] other reagents The reagent R 11 S [illegible]

[illegible]

This study was aided by a grant from the *Swedish Cancer Society* For excellent technical assistance the authors are indebted to Mrs *Karin Reimer* and Miss *Britt Westerdahl*

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KARL ERIK FJELLSTRÖM and CLAES F HÖGMAN

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In a preceding paper (4) a phenomenon has been described consisting of adhesion of human erythrocytes to sensitized human kidney or lung cells This adhesion differed from mixed agglutination in being elicited only in the presence of a heat labile serum factor, referred to below as accessory factor' The heat labile factor was apparently non specific and was present in all normal sera tested This phenomenon showed many similarities to the so-called 'serological adhesion" (6) or "immune adherence" phenomena (12) The first of these two terms has been used here since it seems to cover reactions of similar but not quite identical nature

The present communication is concerned with studies on the thermolabile accessory serum factor and its relation to the complement system

MATERIAL AND METHODS

The tissue material and the technical procedures in the serological adhesion (SA) test have been described in the preceding paper (4)

Preparation of complement (C') reagents and determination of complement activity The symbols R1 R2 R3 and R4 stand for the complement reagents for determination of the first (C'1) second (C'2) third (C'3) and fourth (C'4) complement component respectively The reagents R1 and R2 and ... fractions in addition to R2 were ... filtratio ... pool ...

... the red cells

... was aided by a grant from the *Swedish Cancer Society* For excellent technical assistance the authors are indebted to Mrs *Karin Reimer* and Miss *Britt Westerdahl*

were sensitized (EA) *according to the principles outlined by Kabat & Mayer* (5) by the use of a rabbit anti sheep red cell serum from the State Bacteriological Laboratory, Stockholm Haemolysis was estimated *after 30 minutes at 37° C in a total* volume of 1 25 ml (1 ml of 1 per cent suspension of EA 0 08 ml of serum to be tested 0 08 ml complement reagent and 0 09 ml VB++) The degree of haemolysis was measured in a Beckman B spectrophotometer at 540 mμ The titer of the complement [illegible] haemolysis units (H_{50}) was calculated from the [illegible] the percentage of haemolysis in the different [illegible] ph paper

The glassware used had been cleaned in bichromate sulphuric acid

Immune electrophoresis of some complement reagents was performed according to *Scheidegger* (17) with plasma protein antisera from Behringwerke A G Marburg Germany

RESULTS

The Activity of Fresh Human Serum

To investigate the nature of the accessory factor, kidney and lung cells sensitized with inactivated, antibody-containing serum were used in most experiments by means of the system kidney cells of blood group A and serum containing anti-A The tissue cells sensitized in this way and subsequently washed were incubated with dilutions of fresh serum and examined for their capacity to adsorb group O red cells This reaction, here referred to as serological adhesion (SA), was obtained with dilutions of fresh serum as high as 1/2000 Usually the titre end-point was about 1/200–1/500 Often a marked prozone phenomenon was present, *i e* weaker reactions with serum undiluted or in low dilutions Fresh serum diluted 1/20 gave optimal reactivity Controls with non-sensitized tissue cells were included in the experiments to ascertain that the fresh serum itself was acting only on cells which had been coated with antibody

Influence of EDTA (trisodium ethylene diamine tetra acetate) and some Divalent Cations

The addition of proper concentrations of EDTA inhibited the reactions Under the experimental conditions described, fresh serum in the presence of 1 mM EDTA did not cause any reaction while at concentrations below 0 6 mM incomplete inhibition was obtained With concentrations above 5 mM the cultured cells slipped off the glass The activity of the serum EDTA mixture was restored by addition of a moderate excess of calcium chloride In part, this was also true with magnesium chloride and cobalt acetate (Table 1)

Influence of the Four Classical Complement Components of human serum

The possibility that the accessory factor could consist of some of the complement components was considered The influence of these components upon SA was tested by means of the complement reagents R1,

TABLF 1

The Influence of FDTA Ca Mg and Co upon the SA React on between Group O Red Cells and K Iney Cells of Group A (No 91/C1) Sens t e l w th anti A (No 11) and Subsequently Incubated w th Fresh Group AB Serum (Pool of 1C Samples)

W thout FDTA	320
With 1 mM EDTA	0
W th 1 mM FDTA and 2 mM CaCl	320
2 mM MgCl	160
2 mM C (OOC CH$_3$)$_2$	80

F gures represent rec procal of final dilution of serum showing activity

R2 R3 and R4 The conclusions of the results obtained in about 80 experiments were the following

R1 —
R2 +
R3 +
R4 —
R1 + R4 +

This means that the accessory factor is complex but that it does not consist of all of the four complement components As R2 and R3 according to the criteria were devoid of C 2 and C 3 respectively the results indicate that these components are not necessary for the re action in amounts measurable by immune haemolysis As R1 and R4 gave a negative reaction of themselves but a positive reaction when mixed together the accessory factor may consist of C 1 and C 4

The R3 reagents always had a high activity being about the same as whole serum as judged from serial dilution experiments The R2 reagents on the other hand were usually m ch less act ... n only ... n The ... of the ... C 4 were absent as judged from haemolysis tests They also rapidly lost their capacity to cause the SA reaction during storage In haemolysis experiments it was found that C 4 was com pletely lost after 24 hours of storage at +4° C

In some R2 preparations the SA activity was high enough to permit valid conclusions concerning the role of C 2 in SA The euglobulin precipitates used in the experiments demonstrated in Table 2 were dissolved in an amount of VB corresponding to half the volume of serum used for preparation No haemolysis was obtained with 0 16 ml of the reagents Yet they caused SA in a dilution of 1/64 and 1/32 re spectively This proves that C 2 is not needed for the occurrence of the present type of SA reaction Furthermore in one of these experiments the addition of an excess of C 2 and C 4 did not significantly enhance the SA activity presumably because C 1 was the limiting factor Simi larly a low amount of C 4 seemed to be limiting In the second experi

ment the SA activity was significantly increased by addition of excess C'4 apparently due to the high initial content of C'1 in the R2 reagent The calculated limiting amounts of C'1 and C'4 expressed in H_{50} units were 0 2 to 0.7 in these experiments.

TABLE 2

The SA Activity of Two R2 Preparations Tested by Means of Group AB Kidney Cells (No. 145/61) Sensitized with anti-B (No. 6), and the Influence of Addition of Constant Amounts of R1 and R4 Reagents to all Dilutions of R2.

Addition of R1 and R4 reagents diluted 1 10	Reciprocal of dilution of R2 reagent								Buffer control	The calculated amounts of C'1 C'2, and C'4 (H_{50}) in the final dilution step with a positive reaction		
	2	4	8	16	32	64	128	256		C'1	C'2	C'4
	R2 No I:											
Buffer control	3	4	4	4	2	+	—	—	—	0 7	0	0 6
R1	4	4	4	3	2	+	(+)	—	—	0 4	4 5	5 3
R4	4	4	3	3	2	2	+	—	—	17 0	0 7	0 3
	R2 No II:											
Buffer control	4	4	4	3	+	—	—	—	—	2 8	0	0 2
R1	4	4	4	4	3	3	3	2	—	0 4	4 5	5 0
R4	4	4	3	3	+	—	—	—	—	19 5	0 7	0 2

Key · The strength of the SA reaction · 4 = ++++, 3 = +++, 2 = ++

TABLE 3

SA Reactions Caused by a Mixture of Reagents R1 and R4 in Different Dilutions when Tested with Kidney Cells of Group B (No. 34/61) Sensitized with anti-B (No 6)

		Dilutions of R1					Buffer
		1	5	25	125	625	
Dilutions of R4	1	—	—	—	—	—	—
	5	++	++	++	++	—	—
	25	+	(+)	—	—	—	—
	125	—	—	—		—	—
	Buffer	—	—	—	—	—	—
Whole serum		+++	+++	++++	+++	—	—

The mixture of R1 and R4 preparations had a maximal activity with a proportion of each reagent which varied in different preparations (cf the example in Table 3) Prozone phenomena were frequently observed The titre end-point of a reagent still giving SA corresponded to about 0 2–2H_{50} units of C'1 and C'4

As it has been demonstrated, that antigen-antibody complexes bind the complement components in the sequence C'1 – 4 – 2 – 3 (5, 5a), the experiment shown in Table 4 was conducted From this it was apparent

that one or more factors in R4 must be fixed to the sensitized cells before the factor(s) in R1, if a reaction is to occur The experiment is thus in aggreement with the classical sequence scheme for the reaction of complement components

TABLE 4

Sequential Investigations of the SA Reaction by Means of Reagents R1 and R4 when Tested with Group A Kidney Cells (No 107/61) Sensitized with anti A (No 11) Diluted 1 in 5

		1 Sensitization 2 Incubation with R4 3 Washing × 3 4 Incubation with R1 5 Incubation with group O red cells					1 Sensitization 2 Incubation with R1 3 Washing × 3 4 Incubation with R4 5 Incubation with group O red cells				
		Dilution of R4					Dilution of R4				
		1	10	100	1000	Buffer	1	10	100	1000	Buffer
Dilution of R1	1	++	+++	++	—	—	—	—	—	—	—
	10	++	+++	+	—	—	—	—	—	—	—
	100	+	—	—	—	—	—	—	—	—	—
	1000	—	—	—	—	—	—		—	—	—
	buffer	—	—	—	—	—	—	—	—	—	—

Influence of Other Factors Related to the Complement System

Properdin is one of the co factors involved in the inactivation of C 3 (13) The R3 reagent prepared by inactivation of C 3 at 37° C with zymosan (2 4) simultaneously loses most of its properdin content As R3 caused SA and had about the same activity as whole serum it is not probable that properdin was necessary for the SA reaction Also serum treated with zymosan at 16° C at which temperature properdin is most effectively removed (13), caused SA without measurable loss of activity

Muller Eberhard et al (9 10) have isolated and purified a protein component in human serum named β^1_C on account of its immunoelectrophoretic relation to other β globulins The authors showed that β^1_C can be substituted for R3 prepared from guinea pig serum but not for R3 prepared from human serum Furthermore it has a stabilizing effect upon the EA_{142}[1] complex at 37° C We found by immuno electrophoretic examination that R1 R3 and R4 were devoid of β^1_C As R3 of itself and R1 and R4 in combination are capable of causing SA it is not probable that β_{1C} is of importance for this reaction

Muller Eberhard and Kunkel (11) have recently isolated a thermolabile protein in human serum designated 11 S protein which was found to be related to the complement system The 11 S protein is

[1] EA_{142} means sheep red cells sensitized by anti sheep red-cell antibody and then coated with the complement factors 1 4 2

necessary in the process of haemolysis of sensitized sheep red cells and reacts with these cells before the classical complement components A serum freed from 11 S, called R 11 S, causes very little haemolysis in spite of its full content of C'1, 2, 3, and 4 Two of five preparations of R 11 S, made by us according to the description in (11), did not give SA, whereas the control serum caused SA in dilutions up to 1/160 A third preparation of R 11 S was only partly active in both the SA and haemolysis reactions. two further preparations had retained full activity *Muller-Eberhard* and *Kunkel* (8, 11) have shown that the 11 S component is absent in the R1 reagent This means that the 11 S component and possibly 11 S + C'1 are needed for the SA reaction

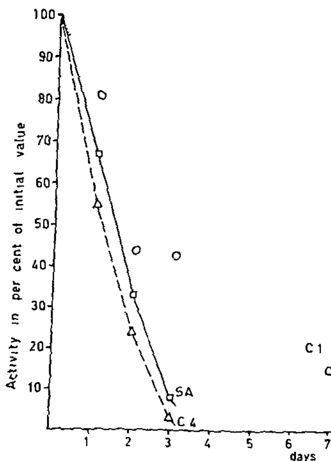


Fig 1
The influence of storage of fresh human serum at 37° C upon the accessory factor of the SA test and upon the complement factors C 1 and C 4

Influence of Storage of Human Serum at Different Temperatures

The capacity of fresh serum to cause SA was sometimes destroyed by treatment of the serum for 5 15 minutes at 56° C After heating for 30 minutes the sera were invariably inactive, but some regained some activity after storage at -30° C and had to be reinactivated

As a rule serum stored at 37° C for 1 or 2 days would lose most of its SA activity Fig 1 demonstrates an experiment in which the residual activity after storage at 37° C measured with the SA reaction is plotted against the content of C'1 and C'4 The curves of the SA and C'4 activities seem to run fairly parallel

Serum stored at 4° C gave SA even after 4 weeks Kept at −70° C serum was active after 6 months

DISCUSSION

The nature of the accessory factor in serological adhesion (SA) occurring in a homologous system in man has been the subject of study in the present investigation As the reagents R2 and R3 were active in high dilutions and as very restrictive criteria were put on these reagents, the conclusion was drawn that C'2 and C'3 in human serum were not necessary for the SA reaction in the system investigated This is in disagreement with previous investigations on the rôle of complement in red cell adhesion and immune adherence (1, 6, 12, 19, 21) in which the whole complement is said to be needed The discrepancy between our results and those of previous authors may be due to differences in the antigen antibody systems or in the complement structure of different species (cf 7) Another possible explanation is the improvement in preparation technique obtained by the use of gel filtration (3) The R1 and R2 preparations were made within about one hour and were used immediately There was no need for addition of heated serum to the R2 reagents as used by some authors (cf 5) It may be stressed that by heating the serum the 11 S component is lost The following facts speak in favour of the accessory factor constituting a combined action of 11 S C'1 and C'4

1 A reagent in which 11 S and C 1 (R1) or C'4 (R4) are absent is inactive but activity is restored by combination of these two reagents

2 If R1 and R4 are tested separately, R4 (containing 11 S + C'1) must be brought into contact with the sensitized cells before R1 (containing C'4) If the reactants be added in the reverse order the reaction will not take place This is in agreement with the sequence scheme for the fixation of C' components in immune haemolysis

3 Of the other known hydrazine-sensitive serum components besides C 4 the possible participation in the reaction of [illegible] scussed [illegible] positive [illegible] minute [illegible] the reaction

4 As C 3 consists of at least two components (15, 16, 18) it can be concluded that only the part which is absorbable with zymosan is not necessary for the SA reaction

5 The lack of C'4 in a euglobulin preparation makes an inactive reagent The parallel deterioration of the accessory SA factor and C'4

necessary in the process of haemolysis of sensitized sheep red cells and reacts with these cells before the classical complement components A serum freed from 11 S, called R 11 S, causes very little haemolysis in spite of its full content of C'1, 2, 3, and 4. Two of five preparations of R 11 S, made by us according to the description in (11), did not give SA, whereas the control serum caused SA in dilutions up to 1/160 A third preparation of R 11 S was only partly active in both the SA and haemolysis reactions: two further preparations had retained full activity. *Muller-Eberhard* and *Kunkel* (8, 11) have shown that the 11 S component is absent in the R1 reagent. This means that the 11 S component and possibly 11 S + C'1 are needed for the SA reaction.

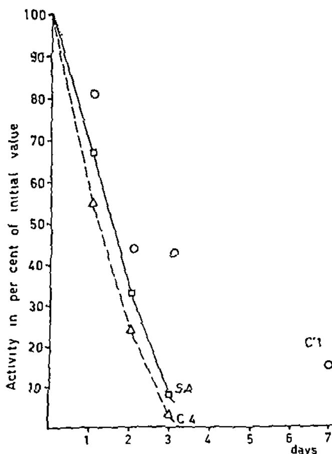


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in whole serum at 37° C and in R2 at 4° C also supports the hypothesis that the hydrazine-sensitive part of the accessory factor is identical with C′4

6 It is known that C′1 acts only in the presence of calcium ions (7) The inhibitory action of EDTA upon SA would thus speak in favour of the participation of C′1 or some other calcium-dependent factor However, the restitution of activity, although incomplete, by the addition of magnesium and cobalt ions to the EDTA-serum mixture seems at first sight to invalidate this assumption But as has been stressed by *Mayer* (7) only minute amounts of Ca^{++} are needed for the action of C′1 and the results may well be explained by leakage of Ca^{++} from the glass or the tissue cells when the EDTA ions have been bound by other divalent cations

7 As no purified preparation of the 11 S component was available to us, it has not yet been fully clarified if the thermolabile component consists of the 11 S component alone, or 11 S + C′1, or C′1 alone

SUMMARY

The nature of the accessory factor of the serological adhesion reaction in a homologous system consisting of human kidney and lung cells, human serum, and human red cells, has been investigated with regard to its relation to the complement (C′) system The C′ reagents used were tested for their activity by a haemolytic system consisting of sensitized sheep red cells (EA)

The accessory factor was complex but did not consist of all of the components of human C′, C′2 and the zymosan-absorbable part of C′3 were not necessary for the serological adhesion reaction

Different types of experiments indicated that the activity is due to a combined action of the 11 S component and/or C′1 and C′4

There was no evidence that β_{1C} or properdin were part of the accessory factor

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MATERIAL AND METHODS

Tissue culture Stationary tube cultures were prepared according to a slight modification (4) of the method of *Youngner* (24) The culture medium consisted of 0 5 per cent lactalbumin hydrolysate and varying concentrations of human group AB serum in Hanks' or Earle's solutions In some experiments Parker medium 199 was used The cells grew in more or less confluent sheets on the wall of neutral-glass test tubes In the experiments the cultures were 4 to 17 days old In most cases about 7 days Cultures with poor growth were discarded

To estimate the approximate number of cells in the cultures 30 kidney or lung tubes from different foetuses were treated with 0 25 per cent trypsin at 35° C for 30–60 minutes to remove the cells from the glass When almost all of the cells were suspended the total number was determined by counting in a Buerker's counting chamber Half the number of tubes were taken before and the other half after incubation in an antibody-containing medium (see below) The number of cells in each tube was found to vary between 50 000 and 220 000 The mean value before incubation was 119 000 and after incubation 143 000

Test sera Serum was obtained from clotted blood of unselected blood donors and from group O mothers who had given birth to infants suffering from haemolytic diseases due to anti A or anti B The sera were used fresh or kept at —25° C without preservatives

Determination of the A antigen of pig red cells The method used was based upon the findings of *Konugres & Coombs* (9) that the "immune" anti A of human sera is active towards the pig A antigen

Human sera containing incomplete anti A were heated at 64° C for one and a half hours to de

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absorbed sera were subsequently used for the determination of the A O groups of pig red cells by means of slide and tube-centrifugation techniques Enzyme treat-

[illegible]

were incubated in 7 × 60 mm

equal volumes of two per cent suspensions [illegible]

incubation at room temperature (20°–22° C)

for agglutination by the [illegible]

Elution of antibodies from coated red cells which had been washed six times in 0 9 per cent saline solution was performed by heating to 56° C for 10 minutes The red cells were removed by centrifugation during 5 minutes in cups containing water heated to 56° C

FROM THE STATE INSTITUTE FOR BLOOD GROUP SEROLOGY, STATENS RÄTTSKEMISKA LABORATORIUM, AND THE DEPARTMENT OF VIRUS RESEARCH, KAROLINSKA INSTITUTET, STOCKHOLM, SWEDEN

INTERACTION BETWEEN HUMAN SERUM AND CULTURED HUMAN FOETAL KIDNEY AND LUNG CELLS

I. *Effect upon Blood Group A and B Antibody Activity*

By

CLAES F. HÖGMAN

Received 6.1.61

The present studies were conducted with regard to problems concerning the mechanism of haemolytic diesease of the newborn due to blood group A and B antibodies. The blood group A and B antigens are present in the cells of many foetal tissues (5, 17). These antigens may absorb antibodies which have passed from the mother's circulation via placenta to the foetus. In this way the red cells of the foetus may be protected against extensive coating by antibodies. This may be one of several possible explanations why the A-B haemolytic disease is comparatively infrequent in relation to the occurrence of antibodies in the mothers. Earlier investigations have mainly been concerned with the rôle of the soluble antigens in this respect (2, 8, 10, 13, 16, 25). In the present study an attempt is made to examine the capacity of living cells grown *in vitro* to inhibit the serological activity of blood group A and B antibodies added to the culture medium.

It is well known that both the antigens and the antibodies of the ABO blood group system are very complex (1, 12, 18, 19, 23). Thus, the so called A^P antigen (also present in the red cells of blood group A pigs) has been described to react only with the "immune" anti-A of man (9, 11, 21) but not with the "naturally" occurring haemagglutinin. It has been found also that the A antigen of human adult leucocytes (3) and buccal cells (20) is almost identical with the A^P antigen. Therefore, some investigations on the relationship between the A^P antigen and the A antigen of human foetal kidney cells are included in the present paper.

This study was aided by a grant from the *Swedish Cancer Society*. For excellent technical assistance the author is indebted to Miss *Britt Westerdahl*.

MATERIAL AND METHODS

... were prepared according to a slight modi-
... 0.5
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...

diseases due to anti A or anti B. The sera were used fresh or kept at —28° C without preservatives.

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Human sera containing incomplete anti A were ...
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... After 1 hour, 1 day, 2, 3, 4, 5, 6
... of at least two tubes was decanted and kept at —25 to
—30° C. In the latter part of the study pools of ...
choice of incubation ...

ously determined In some of the experiments the titrations were repeated The difference between two determinations never exceeded one titre step In others two parallel titrations with different dilutions in the first tube were performed (e g 1/2 1/4, 1/8 etc and 1/3, 1/6, 1/12 etc)

RESULTS

After a short time of contact between the cultured cells and the medium a fixation of antibodies occurred This was demonstrated by mixed agglutination But as the number of cells in each culture was comparatively small the decrease of the titre of homologous antibody in the medium was insignificant at this stage in most of the experiments The aim of the investigation was to see whether a further incubation of the cells would result in a specific removal of antibodies from the culture medium If so, this might indicate a continued production of antigen by the cells On this account the one hour value was considered to represent the most reliable base time

Effect upon anti-A and anti-B Hemagglutinin

In most of the experiments group O serum, thus containing both anti-A and anti-B, was tested Figure 1 shows one example of the experiments in which kidney cultures from group A individuals caused a successive fall in the titre of anti-A, whereas the titre of anti-B remained unaffected or only slightly affected The results of 20 experiments of this kind are summarized in Table 1 A decrease of the titre of

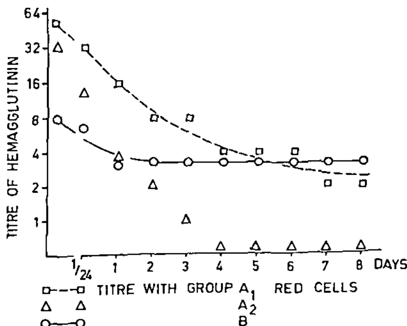


Fig 1
The haemagglutinin titres after incubation of a group O serum with kidney cultures of a group A foetus (No 62/58) The titre values represent geometric mean of two determinations

three titre steps or more was obtained in 6 out of 8 experiments with group A tissue upon the anti A activity and in one out of three experiments with group B tissue upon the anti B activity The titres were determined using 0 9 per cent saline solution as diluent, and the results probably reflect an effect mainly upon antibodies of the complete type

TABLE 1

Decrease in Titre of Haemagglutinin during Cultivation The Difference between one Hour s and 6 Day s Incubation of Kidney Cultures

Blood group of foetus	Decrease of titre Number of titre steps											
	Anti A_1				Anti A				Anti B			
	0	1	2	>3	0	1	2	>3	0	1	2	3
A	1		1	6	1	–	–	7	2	4	2	–
B	2	2	–	–	2	1	–	–	–	1	1	1
O	4	4	–	–	5	2	–	–	4	4	–	–

Figures represent number of experiments

TABLE 2

Decrease in Titre of Haemagglutinin during Cultivation Comparison between Titres Performed in Physiological Saline and in Completing Medium

Blood group of foetus	Titres performed in					
	Physiological saline			Completing medium		
	Anti A	Anti A	Anti B	Anti A_1	Anti A	Anti B
A*	1 8	2 2	1 0	2 0	1 8	1 4
B*	0 4	0 6	1 7	0 6	0 9	2 0
O§	0 5	0 4	0 4	0 6	1 2	0 5

Figures represent titre steps * Mean of 5 experiments § Mean of 7 experiments

In order also to determine incomplete A or B antibodies titration was made in a completing medium (11), either consisting of group AB serum (22) or of the dextran serum solution introduced by *Munk Andersen* (14) The sera used in 15 out of 17 of these experiments were obtained from mothers who had been delivered of ABO incompatible infants in whom the severity of the jaundice required exchange transfusions Also in these experiments the titre of the homologous antibody was affected more than that of the heterologous Table 2 shows an attempt to determine whether antibodies demonstrated by titration in saline were affected to a higher degree than those tested in the completing medium They were not, thus indicating possibly that complete as well as incomplete antibodies were affected in the procedure A further illustration of the problem is given in Fig 2 It is seen that the kidney cells caused a five titre steps' drop of the anti A_1 dextran serum

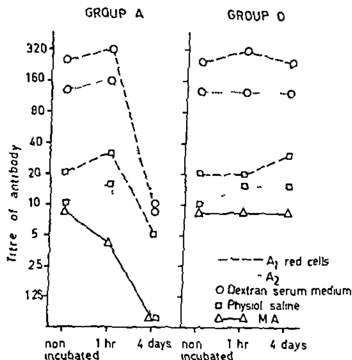


Fig 2
The effect of incubation with kidney cells of groups A and O upon the anti A titre determined by haemagglutination and mixed agglutination (MA)

titre However, in a separate experiment the presence in the serum of an anti-A of "immune" type was demonstrated by addition of an amount of human group A secretor saliva sufficient to inhibit completely the A_1 antibody in saline The remaining titre of incomplete anti-A as determined by testing in dextran-serum solution was the same as before partial neutralization This seems to indicate that the "immune" anti-A of this serum was affected in a different way by the kidney cells than by the group A substance of the saliva

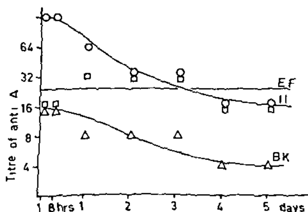


Fig 3
The decreasing effect of kidney cultures (No 185/59) upon the titre of anti A of three group O sera The culture medium contained 50 per cent of the respective serum Titrations were performed in dextran serum solution

In some of the experiments no obvious decrease of the agglutinin titre was observed As is mentioned below this might have been due to a too large amount of antibody added to the culture medium in these cases However the experiment described in Figure 3 illustrates that this explanation was not always true Three different group O sera were simultaneously incubated with kidney group A tissue cultures of the same batch The anti A of the serum with the highest initial titre decreased by three titre steps whereas the serum with lower titre was not affected the titre of the third serum dropped two titre steps

All of the above described results refer to kidney cultures No significant decrease of the haemagglutinin titres were observed in experiments using lung cultures from three group A and two group O foetuses and liver cultures from four group A and one group O foetus

TABLE 3

Decrease in Titre of Blood Group A Antibody Causing Mixed Agglutination after Incubation with Kidney Cultures from one Group A and one Group O Foetus

Test cells	Incubation time	Incubation with kidney cultures Anti A serum in culture medium			
		No 177 group A		No 173 group O	
		10%	50%	10%	50%
Kidney No 177 group A	Non incubated	8	40	8	40
	1 hour	4	40	8	40
	4 days	0	20	8	40
Lung No 177 group A	Non incubated	8	40	8	40
	1 hour	4	40	8	40
	4 days	0	20	8	40

Effect upon Antibodies Causing Mixed Agglutination

In about 50 experiments the culture medium was tested by means of mixed agglutination (MA) In several of these experiments the same batch of cell cultures was used in the MA test as the one used in the preceding absorption experiment Besides cell cultures from other individuals and other organs (lung and liver) were included in the MA tests

Incubation of the antibody containing medium with kidney cells caused a decrease of the titre of the A or B antibodies capable of giving MA reaction The effect was demonstrated by tests on kidney cells as well as on lung and liver cells (cf Table 3) However if lung and liver cells were incubated with the antibody containing medium a performance of the MA tests would leave the antibodies unaffected

If amounts of antibodies larger than 16 mixed agglutinating units [1]

[1] One MA unit means the minimal amount of antibody which under the experimental conditions causes mixed agglutination

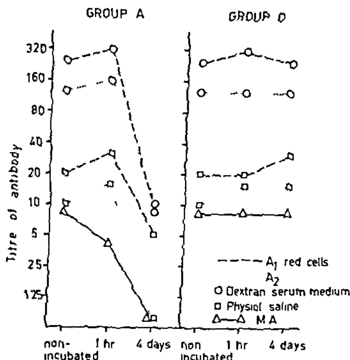


Fig 2

The effect of incubation with kidney cells of groups A and O upon the anti A titre determined by haemagglutination and mixed agglutination (MA)

titre However, in a separate experiment the presence in the serum of an anti-A of "immune" type was demonstrated by addition of an amount of human group A secretor saliva sufficient to inhibit completely the A_1 antibody in saline The remaining titre of incomplete anti-A as determined by testing in dextran-serum solution was the same as before partial neutralization This seems to indicate that the "immune" anti-A of this serum was affected in a different way by the kidney cells than by the group A substance of the saliva

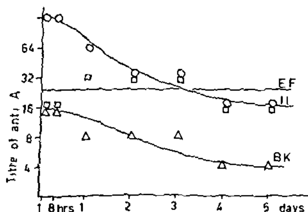


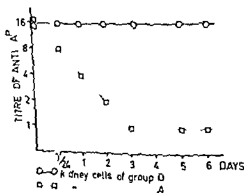
Fig 3

The decreasing effect of kidney cultures (No 185/59) upon the titre of anti A of three group O sera The culture medium contained 50 per cent of the respective serum Titrations were performed in dextran serum solution

were added to the culture medium the decrease of the titre was less obvious (see Table 3)

Relation between the A Antigen of the Foetal Kidney cells and the A^P Antigen

In absorption and elution studies it was found that the A antibody causing mixed agglutination was mainly directed against the A^P antigen A typical experiment is presented in Table 4 This experiment further shows an asymmetric type of cross reaction with group B kidney cells the indicator red cells of group A reacted more strongly than those of group B Although the pig group O cells contained a B like antigen they showed no reaction with antibody coated kidney group B cells whereas the pig group A red cells did react However, in some other tests an A antibody causing MA with only a few cells in the cultures remained after three absorptions As the major part of the group A kidney cells showed an A^P specificity it might be expected that the A^P antibody would be removed from the culture medium by kidney cells of blood group A which was actually the case as demonstrated in Figure 4



DISCUSSION

It has been demonstrated that human foetal kidney cells grown in cell culture have the capacity of lowering the titre of the homologous blood group A or B antibody contained in the culture medium The effect occurred over a period of up to 4 to 6 days It was obtained only with cultures of kidney cells not with lung and liver cells probably because of a smaller number of antigenic receptors in the latter cells This assumption is supported by the previous finding (5) that the number of A or B positive cells giving specific mixed agglutination

TABLE 4

Relation between the AF Antigen and Human Kidney Group A and B Antigen Tested by Means of a Group O serum from a Mother to a Group A Infant with severe Haemolytic Disease. The Serum Prediluted 1/10.

Sensitizing serum (GBJ) absorbed three times with red cells of groups	Haemagglutinin titres against red cells					Mixed agglutination														
						Kidney group A					Kidney group B					Kidney group 0				
	Human			Pig		Human red cells			Pig red cells		Human red cells			Pig red cells		Human red cells			Pig red cells	
	A_1	A_2	B	A	0	A_1	B	0	A	0	A_1	B	0	A	0	A_1	B	0	A	0
Non absorbed	64	64	16	8	8	++++			++++	—	+++	+	—	+++	—	—	—	—	—	—
Pig A	8	2	0	0	0	—	—	—		—	—	—	—	-	—	—	—	—	—	—
Pig O	64	32	4	4	0	++++			++++	—	++	—	—	+	—	—	—	—	—	—
Eluate of sens Pig A red cells	4	2	0	2	0	++	—	—			+	—	—			—	—	—		
Eluate of sens Pig O red cells	0	0	0	0	0		—	—			—	—	—			—	—			

SUMMARY

The interaction between the A B blood group antigens of human foetal organ cells and the corresponding antibodies of human serum has been studied Cell cultures of kidney, lung and liver from foetuses of different ABO blood groups have been incubated with human anti A and anti B of different nature and concentrations

1 A specific effect of blood group A kidney cells upon anti A and of group B cells upon anti B was observed

2 The effect concerned haemagglutinins determined by blood group A_1 as well as A_2 red cells Likewise it concerned antibodies detected by testing in physiological saline as well as in serum or dextran serum medium

3 The A antigen of the foetal kidney corresponded mainly to the A^P antigen and the A^P antibody was inhibited during cultivation with group A kidney cells

4 Cultured lung and liver cells did not cause any reduction of titre This was due probably to a smaller number of antigen carrying cells in these cultures

5 The antibodies of certain sera were not affected Some possible explanations hereof are discussed

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was much smaller in cultures of lung and liver than in cultures of kidney In some experiments the anti-B was reduced even by group A cells (cf Table 2) This may be due to an effect upon A-B cross reacting antibodies It was found that group B kidney cells cross reacted with certain A^P antibodies

The group A antigen present on the red cells of newborn infants is known to react more weakly than the one seen on adult cells (1, 23) Not infrequently the erythrocytes of group A_1 infants are not agglutinated by absorbed sera containing anti-A_1 Therefore, it might be expected that an antibody of anti-A_1 specificity should have been left unaffected by the foetal group A tissue Although such tendency seemed to be demonstrable in some of the experiments (cf Fig 1) the effect upon titres obtained with adult type A_1 and A_2 red cells was often about the same

After partial neutralization with soluble group A substance according to *Witebsky* (22) an antibody of anti-A_1 specificity remains in the serum of certain individuals This antibody can only be detected by the use of completing medium (*e g* AB serum or dextran-serum solution) or by indirect antiglobulin technique The results indicate that some incomplete A or B antibodies may have been absorbed and neutralized by the foetal tissue antigens The A^1 specificity, which is described as one of the "immune" characteristics of group A antibodies, was also characteristic for the kidney group A antigen The titre of anti-A^P was significantly decreased by group A kidney cells

On the other hand, the antibodies of some sera were not affected by the cultured cells It may be assumed that most sera contained a mixture of group A or B antibodies with different subspecificity (18) If the cultured cells lacked one fraction of the relevant blood group antigen and the corresponding antibody fraction existed in the highest titre, any specific effect exerted by the cells on the other antibody fractions would have been masked

Other possible explanation of the failing cellular effect upon the antibodies may concern the technique The cellular metabolism may have been decreased by toxic substances in the culture medium Such toxic substance may (7) or may not be an antibody Furthermore, if the amount of antibody in the medium has been too large a partial removal, if any, may have been too small to be demonstrable in a titration

Certainly, the process of interaction between blood group A-B antigens and antibodies is very complex, and it does not seem possible to draw any general conclusions on the basis of data presented as regards the A-B haemolytic disease of the newborn infant However, in the study of the serological and biological properties of antibodies, *e g* separated by biochemical methods (8), the tissue culture system may prove useful and may contribute to the understanding of the haemolytic disease

FROM THE STATE INSTITUTE FOR BLOOD GROUP SEROLOGY, STATENS RATTSKEMISKA LABORATORIUM, AND THE DEPARTMENT OF VIRUS RESEARCH, KAROLINSKA INSTITUTET, STOCKHOLM, SWEDEN

INTERACTION BETWEEN HUMAN SERUM AND CULTURED HUMAN FOETAL KIDNEY AND LUNG CELLS

2 *Observations on Cells Sensitized with Blood Group Antibodies*

By

CLAES F HÖGMAN

Received 6 i 61

In previous papers (5, 6) the presence of antigens considered to be blood group A and B antigens was demonstrated on the surface of cells derived from human foetal kidney, liver, spleen, lung, heart and skin When exposed to certain human sera these cells, which were propagated *in vitro*, acquired the capacity to adsorb specifically human erythrocytes ("mixed agglutination" (1))

Kidney cells grown in a medium containing blood groups A and B antibodies were able to reduce significantly the titre of the homologous antibody when certain sera were used (4, 8) The removal of antibodies from the culture medium was due probably to cellular activity. In the present investigation this question was studied by determination of the period during which sensitized and washed cells still showed the presence of antibodies on the cell surface This was examined by the mixed agglutination technique

MATERIAL AND METHODS

The culture medium consisted of balanced salt solution (BSS, Hanks or Earle) with 0 5 per cent lactalbumin hydrolysate and 10 or 20 per cent human serum from group AB donors In some of the experiments Parker 199 [illegible]

This study was aided by a grant from the *Swedish Cancer Society* For skilful technical assistance the author is indebted to Miss *Britt Westerdahl*

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usually completely negative, the time needed to reach this stage varying between 4 and >72 hours After 20 24 hours at 4° C the reactions were almost as strong as at the beginning of the experiment (cf Table 1) Apparently, positive reactions disappeared at much lower rates at this temperature

The effect of dilutions of the sensitizing serum, representing a varying degree of sensitization of the cells, is demonstrated by the example in Table 3 It is seen that the cells sensitized with low serum dilutions were positive for a longer period than cells sensitized with diluted serum

TABLE 2

Incubation Period Necessary for the Disappearance of the Capacity to Cause Mixed Agglutination between Previously Sensitized Kidney or Lung Cells and Erythrocytes of Corresponding Group

	Tissue from foetuses*	In vitro age of tissue at start of experiment	Sensitizing serum			Mixed agglutination reaction	
				Dilution§	Blood group	Last positive reaction after hrs	First negative reaction after hrs
1	K 95 59	9	K B	1 2	O	45	>45
2	K 81/58	20	G B.J	1 5	O	24	48
3	K 97/58	16	G B.J + I A + 4477	1 10	O	14	24
4	K 104/58	7	G B J + I A + 4477	1 /10	O	14	24
4	K 104/58	7	G B.J + I A + 4477	1 10	O	14	24
5	K 133 58	8	I A + C	1 5	O	12	16
6	K 38/60	7	I I	u	O	16	24
7	K 38 60	7	I I	1 5	O	8	16
8	K 38/60	7	I I	1 20	O	1	4
9	K 191/59	7	I I	u	O	24	48
10	K 191/59	7	I I	1 10	O	8	16
11	K 191/59	7	E F	u	O	8	16
12	K 191/59	7	E.F	1 10	O	4	8
13	K 191/59	7	B K	u	O	8	16
14	K 191/59	7	U H	u	O	4	8
15	K 58 59	6	N	u	O	4	12
16	K 163/58	12	KS 4/11	1 5	B	16	20
17	K 187/58	11	KS 4/11	1 5	B	12	16
18	K 68 59	14	KS 4/11	1 2	B	8	16
19	L 133 58	8	I A + C.	1 5	O	12	16
20	L 60/59	15	B T	1 3	O	12	16
21	K 45/59	8	K	u	O	72	>72
22	K 63/59	8	K	u	O	24	40
23	K 95/58	7	I A	1 5	O	20	24
24	K 132 58	9	I A + C	1 10	O	12	16
25	K 45 59	6	KS 5/11	u	A	12	16
26	K 75/59	13	F	1 2	A	24	48
27	K 143 60	10	E	u	A	24	48
28	K 143/60	10	E	u†	A	24	48
29	L 143/60	10	E	u	A	24	48
30	L 143/60	10	E	u†	A	24	48
31	L 143/60	20	F	u	A	24	48
32	L 63/59	11	K	u	O	16	20
33	L 63/59	11	E.	u	A	16	20

* No 1 20 tissue of group A no 21 33 tissue of group B K = Kidney L = Lung
§ u = undiluted † Serum inactivated for 30 min at 56° C.

Experimental Cultures were sensitized by incubation for 30 minutes at 35° C with 0.5 ml volumes of a serum or serum dilution known to cause mixed agglutination of tissue cells and erythrocytes. After decantation of excess serum the cultures were washed three times in balanced salt solution (BSS) or phosphate buffered saline, pH 7.2–7.4 (PBS). Subsequently the cultures were immediately reincubated with culture medium not containing the relevant antibodies. After intervals of as a rule 1 hour, 4, 12, 16, 20, 24 and 48 hours the medium of three (in some tests of two) tubes was decanted and replaced by 0.5 per cent suspensions of group A_1, B or O erythrocytes in PBS. The culture tubes were again stoppered and kept stationary at an angle of about 5° from the horizontal plane with the tissue layer downwards. After one hour the erythrocytes had formed a sediment on the cell culture layer. The tubes were rocked gently four or five times to loosen the erythrocyte sediment upon which they were placed in a rack in a vertical position. After another hour the free red cells had sedimented on the bottom of the tubes and the cell culture layer was inspected under the microscope (magnification 16 × 8). Mixed agglutination of cultured cells and erythrocytes was observed and the reactions were recorded as follows: ++++, more than 50 per cent of the cultured cells were involved in mixed agglutination, +++, 5–50 per cent positive, ++, 0.5–5 per cent positive, and + less than 0.5 per cent positive.

The portions of culture medium obtained from the sensitized and reincubated cultures were subsequently titrated to determine the presence, if any, of anti-A and anti-B haemagglutinins. Two-fold dilutions were prepared. To 0.5 ml volumes of the dilutions of the culture medium 0.05 ml of a 20 per cent suspension of A_1, A_2 and B red cells was added. After two hours' incubation at room temperature the tubes were read before and after centrifugation at 2000 rpm for 2 minutes. In some experiments the antiglobulin test was performed and in others the red cells were suspended in the dextran-serum solution introduced by *Munk Andersen* (12) and read after further centrifugation.

RESULTS

Effect of Incubation Time and Temperature upon sensitization of cells

Two typical examples of these experiments are shown in Table 1 and a summary is given in Table 2. After one or four hours of incubation at 35° C of the antibody-coated and washed, cultured cells a majority of these were capable of causing mixed agglutination (MA). Later, weaker reactions were often observed and in most of the experiments only few of the cells would adsorb erythrocytes after 8–16 hours. After incubation for 20–24 hours at 35° C the reaction was

TABLE 1

Mixed Agglutination Reactions after Sensitization of Human Kidney Cells and Incubation

Incubation at 35° C hours	Kidney no 101/58 group A, Serum G B J dil 1/20			Kidney no 95/58 group B, Serum I A dil 1/5		
	Indicator red cells of groups					
	A_1	B	O	A_1	B	O
1	++++		—	+	+++	—
12	+++	—	—		++	—
16	++	—	—		++	—
20	+	—	—		+	—
24	—	—	—	—	—	—
+4° C for 20 hours	++++	—	—			

TABLE 4

Mixed Agglutination Reactions after Incubation at 35° C Using Different Culture Media

Incubation time in hours	Experiment no 7 Lung cultures group A				Experiment no 13 Kidney cultures group B					
	Medium Ia		Medium II		Medium Ia		Medium Ib		Medium II	
	In indicator red cells of groups									
	A	O	A	O	B	O	B	O	B	O
1	+++	—	+++	—	+++	—	++++	—	++++	—
4	+++	—	+++	—	+++	—	++++	—	++++	—
12	+	—	+++	—	+++	—	+++	—	++++	—
16	—	—	+++	—	++	—	++	—	++++	—
24	—	—	+++	—	++	—	++	—	+++	—
40	—	—	+++	—	—	—	+	—	+++	—

Key

Ia = 20% AB serum
80% Simms' solution
Ib = 20% absorbed O serum
80% Simms solution
II = 100% Simms solution

Antibodies in the Culture Medium

It migh be expected that some of the antibodies on the surface of the sensitized cells could be eluted again and appear in the culture medium Anti A or anti B haemagglutinins were indeed demonstrated in 11 out of 12 experiments in which sensitizing sera with high titres were used, provided the sensitivity of the haemagglutinin tests was enhanced by centrifugation A further slight increase of the titre of antibody was observed by the indirect antiglobulin technique in one out of three experiments The highest titre values were seen in dextran-serum solution (cf Fig 1)

The effect of
sensitive metho
even in the thir ... serum containing very potent antibodies was used for sensitization After incubation a rise by three titre steps occurred within the first hour, probably due to elution from the sensitized cells upon which a steady state in the titre, or a slight decrease, was seen

In another experiment demonstrated in Fig 2 the culture medium was changed three times after the washings and the titre of eluted antibody was determined After 30 minutes' reincubation the titre rose by two steps but decreased successively when the medium was repeatedly changed Elution appearing after this procedure seemed to occur more slowly However, this repeated change of culture medium did not affect the rate of disappearance of antibodies from the cellular surface as measured by mixed agglutination

TABLE 3

The Effect of Dilution of Sensitizing Serum and of the Presence of Serum in the Culture Medium upon the Mixed Agglutination Reaction

Time of incubation	Incubation medium					
	20 % AB serum 80 % medium 199			100 % medium 199		
	Dilution of sensitizing serum (1 1)					
	U	1 5	1 20	U	1 5	1 20
1	++++	++++	+++	++++	++++	++++
4	+++	++	—	++++	++++	+++
8	+++	+	—	+++	+++	+++
16	+	—	—	+++	+	—
24	—	—	—	++	+	(+)
48	—	—	—	++	—?	—

U = undiluted (Cf Table 2 experiments 6 7, and 8)

In some of the experiments the cells were sensitized again at a time when they showed negative reaction after incubation This resulted in a capacity to adsorb red cells which was as strong as before, *i e*, the cells had not become refractory to sensitization during cultivation

In two experiments with kidney and lung tissue from a group B foetus it was shown that inactivation of an anti-B serum for 30 minutes at 56° C did not influence the period during which the cells showed mixed agglutination (cf Table 2b)

Effect of Different Culture Media upon Sensitization of Cells

In nine experiments sensitized cells were incubated with two different culture media, one consisting only of BSS with dextrose, the other containing also 20 per cent of group AB serum The capacity to adsorb erythrocytes persisted much longer when the cells were incubated in the salt medium than in the protein medium In the BSS medium the cultures could not be maintained for more than 48 hours This period was not long enough for a negative reaction to occur, but a weakening of the positive reaction was noted in some of the cases (cf Table 4)

When Parker medium 199 was used the reaction decreased or vanished although it persisted for a longer period than had been the case when a serum containing medium was used (Table 3)

As the presence of soluble blood group substance in the AB serum could affect the result, group O serum absorbed with group A_1B erythrocytes washed three times, was also tested To remove erythrocyte stromata the serum was passed through a Seitz filter No 6 In two further experiments serum from a group AB non secretor was used for incubation The results were in good agreement with those in which the pool of AB serum was used

It might be concluded from these experiments that elution occurs from the sensitized cells and that an equilibrium seems to be established rapidly after washing and reincubation. Subsequently the antibody level of the culture medium may show a slow decrease but still antibodies may be demonstrable even when the mixed agglutination reaction on the cultured cells is negative.

DISCUSSION

Mixed agglutination (MA) is the specific adhesion between two different types of cells, *e.g.* organ tissue cells and erythrocytes (1, 5, 6), considered to be due to antibodies directed against an antigen common to both types of cells. If a living tissue cell is sensitized and later exposed to a medium devoid of the antibody in question, the period of persistence of capacity to cause MA would indicate persistence of antibodies on the cellular surface. To permit of valid conclusions being drawn, however, it is necessary to exclude serological adhesion (9, 10), which requires an accessory factor sensitive to heat and ageing (2) as a cause of the reactions. By controls using red cells of group O and by experiments using heat inactivated serum it was ascertained that the reactions observed really were of the MA type.

During incubation the sensitized cells successively lost their capacity to cause MA. The reaction disappeared after varying periods depending on different factors.

Apparently the initial degree of sensitization was of importance. This was sh[illegible] reaction o[illegible] (cf. Table [illegible] partly exp[illegible] different results obtained with different sera.

The disappearance of the MA capacity was not due to a loss of antigenic receptors. At a time when the cells were desensitized after incubation, it was possible to sensitize these again and to obtain MA. So it seems likely that the antibodies previously fixed to the membranes of the cells by means of a passive or active mechanism had either disappeared from the membrane or had been changed in such a way that their original serological activity had been destroyed.

If all of the antibodies had been gradually eluted from the cells during incubation by means of a simple physical process, the result should have been a successively rising titre of antibodies in the culture medium occurring parallel to the weakening of the MA reaction. But this was not the case. It is true that antibodies were found in the incubation medium in most of the experiments but usually only in small amounts and with a maximal titre occurring already in the one hour sample, i.e. as a rule long before the MA reaction was negative. The titre then persisted at the same level or decreased by a few titre steps. These facts together with the fact that cultured cells may completely

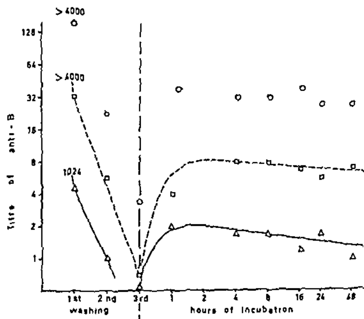


Fig 1.

Titre of anti-B haemagglutinin in washings and culture medium at incubation after sensitization of kidney and lung cells with a serum containing a strong anti B, geometric means of four experiments

Key: △——△ Titre in saline after two hours' sedimentation at room temperature
□ - - □ Titre in saline after subsequent centrifugation
○ ○ Titre in dextran-serum solution after centrifugation

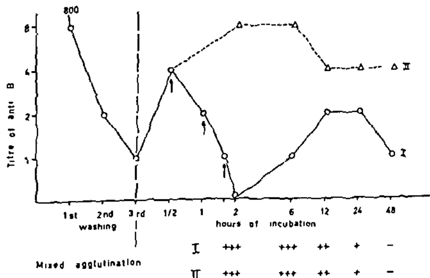


Fig 2

Titre of anti-B haemagglutinin in washings and incubation medium after sensitization of lung cultures from a group B foetus (No 148/60) with a strong anti-B serum The culture medium consisted of 5 per cent absorbed group O serum and 0 5 per cent lactalbumin hydrolysate in BSS Earle During the first 1¾ hours of reincubation the culture medium was changed thrice as indicated by arrows in the figure The titres were read after two hours' incubation at room temperature and subsequent centrifugation

I. Change of culture medium II· Control No change of culture medium

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remove blood group antibodies which have been previously added to the culture medium (4, 8) seem to rule out the possibility that the phenomenon could have been caused by elution only

Other "non-specific" eliminative processes are conceivable Extra cellular enzymes produced by the cells might destroy the antibodies However, the frequent persistence of free antibodies in low titre seems to rule out this possibility

The differences in the rate of elimination observed at different incubation temperatures and with different culture media probably reflect variations in the rate of cellular metabolism The most rapid disappearance of the MA capacity was obtained if the culture medium contained serum The possible presence of serum substances causing inhibition of the reaction should be taken into account It is known that sera from group AB persons, irrespective of their secretor state contain small amounts of AB substance (3) However, the fact that a similar effect was found when an absorbed group O serum replaced the AB serum rules out the possibility that AB substance could have been responsible for the disappearance of the reaction

The most likely explanation of the results seems to be a specific eliminative process After having been fixed to specific antigenic receptors on the cell surface the antibodies may be inhibited or destroyed because of a cellular action Thereby, the antibody molecules may be incorporated in the cell by pinocytosis (11) Another possibility is that they are neutralized by an antigenic substance produced by the cell and that the serologically inactive complex is finally sheed from the cell surface

SUMMARY

Human cells derived from foetal kidney and lung were propagated *in vitro* The cells were sensitized by means of human sera containing blood group antibodies They acquired the capacity specifically to adsorb erythrocytes of the corresponding blood group (mixed agglutination)

By reincubation of sensitized cells in a medium devoid of specific antibodies this capacity disappeared after a period varying from 4 to >72 hours partly depending on the sensitizing serum

The time required for the capacity of causing mixed agglutination to disappear also depended on the type of medium and the temperature of incubation indicating that the rate of cellular metabolism could be a factor of importance

This loss of haemadsorption capacity was probably due to an elimination or inactivation of antibodies by some activity of the cell

intravenous injection of a normal human serum This antiserum was especially suitable because only a few α 2 precipitation lines developed in addition to the Gc line For the second analysis either a pool of antisera from 10 rabbits immunized as described above or a horse anti human serum (No 13414) from the Pasteur Institute in Paris was used

Agar Gel Electrophoresis

The haptoglobin (Hp) type was determined by means of agar gel electrophoresis as described by *Hirschfeld* (3)

RESULTS

Figure 1 shows the 3 Gc types as seen with the single rabbit antiserum (792) It can be deduced from the shape of the precipitation arcs that the fast moving type (Gc 1 1, serum G) as well as the slow moving type (Gc 2 2, serum P) are electrophoretically homogeneous components The heterozygote (Gc 2 1, serum E), however, has an extended precipation band, which in some cases is seen as a double curved line At the lower part of the figure is shown the result of analysis performed on a mixture of equal parts of serum G (type 1–1) and serum P (type 2 2), this mixture has exactly the same appearance as the heterozygotic serum E (type 2 1) and cannot be distinguished from this The shape of the precipation line seen with the natural type 2 1 as well as with the mixture of the two homozygotic sera indicates that the fast and the slow moving components are antigenically related but their electrophoretic mobilities distinctly different These three serum samples (G, P, and E) as well as the mixture of serum G and serum P have been used as controls in each set of analysis A limited number of sera from each group were also analyzed in a Michaelis buffer (pH = 8 6 ionic strength 0 05 in agar), and it was shown that this change in buffering medium had no influence on the actual typing of these sera

TABLE 1

The Gc Type Distribution Obtained by Determination of the Gc Types of 292 Swedish (Hirschfeld (6)) and 126 Danish Sera Respectively

	Gc 1 1	Gc 2 1	Gc 2 2	Total
Danish sera	73 (58 %)	47 (37 %)	6 (5 %)	126
Swedish sera (Hirschfeld (6))	161 (55 %)	108 (37 %)	23 (8 %)	292

The χ^2 test shows no significant difference between the distributions (χ^2 = 1 35 d f = 2 0 5 < P < 0 6) Calculated gene frequencies Gc^1 = 0 77 Gc^2 = 0 23

Table 1 shows the result of the Gc type determination on the 126 samples Typing was possible in all sera without difficulties From the distribution obtained with the Danish sera the gene frequencies were calculated Gc^1 = 0 77 and Gc^2 = 0 23

FROM THE DEPARTMENT OF BIOPHYSICS, STATENS SERUMINSTITUT, COPENHAGEN

IMMUNOELECTROPHORETIC ANALYSIS OF THE GC-TYPES IN HUMAN SERA

1. *Determination of the Gene Frequencies in the Danish Population*

By

BENDT MANSA

Received 29.xi.61

Immunoelectrophoretic analysis, originally described by *Grabar* & *Williams* (2), enables separation of normal, human serum in at least 20 components. The micromethod introduced by *Scheidegger* (7) gives corresponding results. By placing the point of application 20 mm's from the cathode end of the agar plate and prolonging the time of electrophoresis, *Hirschfeld* (4) obtained an improved separation of especially the α-2-globulins. With this method, *Hirschfeld* (4) showed that human sera belong to one of 3 distinctly different types due to characteristic differences in electrophoretic mobility of an α 2 component the group-specific protein (Gc). The types are antigenically closely related, in that absorption of antiserum with each individual type will remove antibodies against all types (4). As will be described later in this paper (see also fig. 1) the three types of sera each show characteristic precipitation bands with the corresponding antibodies. It has been established that the Gc-types are hereditarily determined by the alleles, Gc^1 and Gc , which give the homozygotes Gc 1-1 and Gc 2-2 and the heterozygote Gc 2-1 (6). The well known haptoglobin (Hp) system (8) is of a similar hereditary character, but independent of the Gc-system (5). This paper presents the preliminary results of an investigation of the distribution of the group specific protein types in sera from normal adults in the region of Copenhagen in Denmark.

MATERIAL AND METHODS

For this study serum samples taken at random from 126 normal adults have been used.

ld (4) using the paper electrophoresis equip employed with the following modifications t (w/v) was used throughout and the point of application was placed 25 mm's from the cathode end of the agar plate.

All results are derived from double analysis. For the first determination an antiserum from a single *rabbit* was used. This antiserum (792) was prepared by repeated

The haptoglobin (Hp) types determined are given in Table 2 where the Hp types for each of the Gc-groups also are shown. The gene frequencies determined were $Hp^1 = 0.36$ and $Hp^2 = 0.64$. Of the possible combinations all are present in the material, except the very rare combination Gc 2–2, Hp 1–1. The difference between the electrophoretic mobilities of the Hp 2-1 and Hp 2–2 complexes with hemoglobin is small. In a few cases, where the electroendosmosis was less than normal, it was not possible to differentiate with certainty between these types. In such cases the agar gel electrophoresis was repeated and generally the types could now be determined without difficulty.

TABLE 2

The Number of Sera Belonging to each of the Hp (Haptoglobin) Types

	Total	Gc 1-1	Gc 2-1	Gc 2-2	Galatius-Jensen
Hp 1-1	18 (14 %)	12 (17 %)	6 (13 %)	0	328 (16.03 %)
Hp 2-1	54 (43 %)	33 (45 %)	19 (40 %)	2 (33 %)	967 (47.26 %)
Hp 2-2	54 (43 %)	28 (38 %)	22 (47 %)	4 (67 %)	751 (36.71 %)
Total	126	73	47	6	2046

The Gc-type distribution is compared with the results published by *Hirschfeld* (6) obtained by typing a similar Swedish material consisting of 292 serum samples (Table 1). The Danish material shows no statistically significant difference from the Swedish ($0.5 < P < 0.6$).

With respect to the distribution of the Hp-types the sera used in this investigation can be regarded as normal, *i.e.* no statistically significant deviation from the previously demonstrated distribution in the Danish adult population [*Galatius Jensen* (1), Hp 1–1 = 328 (16.03 per cent), Hp 2–1 = 967 (47.26 per cent), Hp 2–2 = 751 (36.71 per cent)] can be demonstrated ($0.3 < P < 0.4$). Further, the distribution of Hp types among the sera belonging to the individual Gc-types is compared with the Hp-type distribution in the total number of sera, the Gc-groups 2-1 and 2–2 are taken together. Again no statistical deviation is found ($0.4 < P < 0.5$). Thus, the two hereditarily determined α 2 protein systems, Gc and Hp, are completely independent of each other.

Gc type determination of a few sera from mother/child agrees with the hypothesis of two alleles without dominance (6). However, at present the family material examined is too limited for confirmation of the inheritance of these markers.

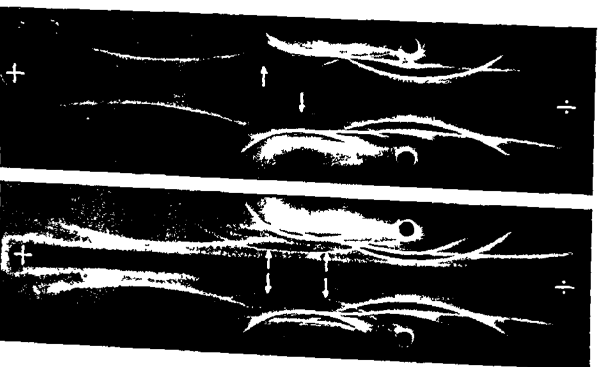


Fig 1

Determination of the 3 different types of group specific protein by means of a selected antoserum (792) The lower part of the figure shows the band obtained after mixing equal parts of serum G (type 1-1) and serum P (type 2-2)

BRIEF REPORT

DOES THE CROSSING FROM PENICILLIN SENSITIVITY TO PENICILLIN RESISTANCE PRODUCE CHANGES IN THE ANTIGENIC PATTERN OF STAPHYLOCOCCUS AUREUS?

By *Birgitta Norkrans* and *Kurt Bertrandsson*

Stern & Flek (3) stated that agglutination tests had disclosed no changes in the

plate technique (4) and one cell cultures resistant strains were produced which had relatively low resistance capacity i e 2 µg/ml in the first two and 40 and 80 µg ml respectively in strains 316 and S 169 The parent strains were sensitive to 0 001 µg ml of penicillin

The bacteria were grown at 37° C and harvested after 72 hours by centrifuging

served by merthiolate 1 10 000 and stored in a freezbox

The antigen solutions for the agar diffusion test were prepared in a glass homogenizer by manual grinding for 10 minutes Intact cells and cell wall fragments were separated by centrifuging The supernatant i e the antigen solution presumably consisted mainly of protoplast antigens Gel diffusion tests were performed as recommended by *Ouchterlony* (2) with three basins in an agar plate using 0 75 per cent Ionagar No 2 (Oxide Ltd London) The test Petri dishes were kept in a moist atmosphere to avoid refilling Readings were made daily for 10 days

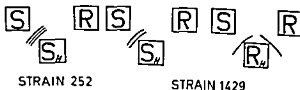


Fig 1

Fig 1 Precipitation pattern of strains 252 and 1429

S = sensitive strain S_H = antiserum against heat killed S
R = resistant strain R_H = antiserum against heat killed R

Received 24 iv 62 *from the Department of Botany University of Gothenburg Sweden*

SUMMARY

Immunoelectrophoretic analysis has been used to determine the Gc (group-specific) protein in human sera The distribution of the Gc types of 126 serum samples taken at random from normal Danish adults is presented and found to be essentially identical to that of a comparable Swedish investigation The gene frequencies from our data are $Gc^1 = 0\ 77$ and $Gc^2 = 0\ 23$ Determination of the haptoglobin types of the sera was also carried out No association between the two hereditarily determined α-2-proteins of the Gc- and Hp systems could be demonstrated

ACKNOWLEDGEMENTS

Thanks are due to Mr *A Birch Andersen* Head of Department of Biophysics for valuable discussions and help during the preparation of the manuscript The statistical analysis were carried out by cand polit *S Olesen Larsen* Statistical Department Statens Seruminstitut His valuable help is gratefully acknowledged Thanks are due

nsic Medicine Copen
number of sera Miss
technical assistance
and expert handling of photographic material

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PROFESSOR TAGE KEMP, M.D., for many years chief editor of Acta Pathologica et Microbiologica Scandinavica, has notified the editorial committee of his resignation from the post for reasons of health

As early as in 1920 Professor Kemp became associated with the daily management of the journal as editorial secretary, since the death in 1941 of Professor Oluf Thomsen Professor Kemp has been chief editor

Always displaying the greatest interest and consideration Professor Kemp has taken part in and attended to the daily management of the journal for a period covering 33 years Notwithstanding difficulties often under trying circumstances, particularly during World War II, the journal has made great progress under his firm leadership during which period it has attained international recognition now being a widely distributed scientific paper

The editors unite in expressing their gratitude to Professor Kemp

Some of our results are shown in Fig 1 Only the sensitive antiserum to strain 252 was produced whereas both sensitive and resistant antisera were prepared against the others In strain 252 acquirement of penicillin resistance seemed to involve a loss of the heat stable protoplast antigen(s) present in the sensitive strain or a change in them Similar changes occurred in the antigenic pattern of the other strains *even if not as striking as those in strain 252 Consequently these* prelimi nary results indicate that changes do in fact take place in the antigenic pattern of penicillin sensitive staphylococci when they aquire penicillin resistance Moreover these changes can be revealed by the double diffusion agar technique

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THE DEPARTMENT OF HISTOLOGY, UNIVERSITY OF UPPSALA, SWEDEN (HEAD: PROF. MARTIN WRETE, M.D.)

THE INFLUENCE OF ANTIGEN ADMINISTRATION ON THE LYMPHOCYTES OF THE LIVER AND EPIDERMIS

By

KARL ERIK FICHTELIUS

Received 12 xii 61

Lymphocytes are known to comprise approximately 10 percent of the cells in the liver (*Osogoe & Awaya* 1960). Although nothing definite is known about their functional significance there, it has been suggested by *Horu, Ilio & Sugimoto* (1950) that "the lymphocyte infiltration in the Glissonian capsule is the display of the lymphocyte-regulating function of the liver and at the same time the realization of the transmission process of the lymphocytes from the blood to the lymph". A similar view was advocated by *Fichtelius & Diderholm* (1959a) who suggested that a large scale recirculation of lymphocytes takes place via the liver and its lymph.

In a number of experiments transfused lymphocytes have been traced to the liver of the recipient. The literature on this subject up to 1960 has been reviewed by *Diderholm* (1961). Thymus and lymph node lymphocytes transfused to recipients immunized with typhi H antigen were recovered in the liver to a lower degree when lymphocytes came ... recovered in the liver to a lower degree when the thymus lymphocytes came from sensitized donors than when they came from non sensitized donors (*Fichtelius* 1959b). Four days after the administration of P^{32} and sheep erythrocytes to guinea pigs the liver DNA activity was lower than in similarly treated animals receiving saline instead of sheep erythrocytes (*Fichtelius, Diderholm & Stillström 1960*). A possible explanation of these observations is that lymphocytes to a certain extent are normally destined for the liver. When antibody production is increased and lymphocytes are needed elsewhere they are either drafted off from the liver or never reach this organ. This conclusion cannot be drawn, however, until it has been shown that the turnover of DNA in the liver is not decreased by antigen administration.

The work has been supported by the Swedish Medical Research Council.

for his great efforts for so many years in which his achievements have been of invaluable benefit to Scandinavian Pathology and Microbiology

The task of Chief Editor has been assigned to Professor K A Jensen M D , The Institute of General Pathology, University of Copenhagen

THE DEPARTMENT OF HISTOLOGY, UNIVERSITY OF UPPSALA SWEDEN
(HEAD PROF MARTIN WRFTE M D)

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… of Swedish Medical Research Council

17*

Thymus lymphocytes were transfused from normal rats to rats immunized with typhi H antigen (*Fichtelius & Diderholdm 1959b*) The transfused cells appeared to accumulate in the liver to a greater extent in the immunized recipients than in the normal recipients These findings do not contradict the typhi H and dinitrochlorobenzene experiments A lower tendency to accumulate in the liver may apply only to lymphocytes which have been in contact with antigen during a certain stage of their development, or according to *Burnet* (*1959*), "lymphocytes of a certain clone" As a consequence of antigen administration the unlabelled lymphocytes formed within the immunized recipients may be drafted off from the liver or may reach this organ to a lower extent, thus giving rise to a real or apparent accumulation of transfused, labelled and normal lymphocytes in the liver

In the following experiment an attempt was made to confirm the observation of the relatively low radioactivity in liver DNA four days after the administration of P^{32} and antigen (*Fichtelius, Diderholm & Stillstrom 1960*) using another antigen and time schedule The eventual disturbing influence of antigen administration on P^{32} incorporation into liver DNA was checked in experiments in which the animals were exposed to P^{32} for only 3 hours, during which time labelled lymphocytes could not reach the liver to any great extent Since some lymphocytes appear to be normally destined for the epidermis (*Fichtelius, Danielsson & Hallander* 1960) this organ was included in the investigation

THE RADIOACTIVITY OF DNA IN LIVER AND EPIDERMIS AFTER ANTIGEN ADMINISTRATION AND SHORT TIME EXPOSURE TO P^{32}

Problem

Is an antigenic influence demonstrable on P^{32} incorporation into the DNA of liver and epidermis?

TABLE 1

	Number of animals	Average weight of group	Treatment on 1st day of exp	Time of P^{32} injection* after 1st day treatment
Group Ia	16	216 g	Primary Immunization §	2 days
Group Ib	11	250 g	1cc phys saline inj subcu	2 days
Group IIa	16	228 g	Primary Immunization	11 days
Group IIb	15	238 g	1cc phys saline inj subcu	11 days
Group IIIa	15	226 g	Primary Immunization	21 days
Group IIIb	16	209 g	1cc phys saline inj subcu	21 days

Note All injections were carried out at 9 am on the way specified Animals were sacrificed 3 hours after P^{32} injection

* 0 3uC P^{32} as orthophosphate per g body weight injected subcutaneously

§ See below

Method

One month old male guinea pigs with an average weight of 220 g at the start of the experiment were used. The animals were divided into 6 groups and were treated according to the schedule in Table 1.

Primary immunization: 0.5 cc agar at 50° C and 0.5 cc typhi H antigen containing 10^8 dead bacteria was mixed in a syringe and injected subcutaneously. The reliability of this immunization technique was checked on a limited number of animals by means of antibody titration which gave satisfactory results.

(The titrations were carried out by Dr. *Astrid Fagraeus* at the State Bacteriological Laboratory in Stockholm.)

Examination of the animals: After the animals were sacrificed with ether the wet weight was determined for the liver. Epidermis was separated according to *Baumberger, Suntzeff & Cowdry* (1941, 42) with 0.32 N NH_4OH. According to *Lundin's* method (1958) DNA was isolated from part of the liver and epidermis. Free P was also isolated according to *Lundin* (1958)[1]. The phosphorus of these fractions was determined according to *Gomori* (1942) and the radioactivity according to *Lindberg* (1946).

The number of impulses per minute per mg DNAP in per cent of the number of impulses per minute per mg free P was calculated and may be used as a relative measure of the DNA turnover in different organs (compare *Andreasen & Ottesen* 1944).

RESULTS

Statistically significant differences were *not* found between the figures for DNA turnover in liver and epidermis in the animals which received typhi H antigen and those which received saline (see Table 2.).

No antigenic influence on P^{32} incorporation complicating the interpretation of the next experiment could thus be demonstrated with the method used here.

TABLE 2

Impulses per Minute per mg DNAP in per Cent of the Impulses per Minute per mg Free P

	Liver	Epidermis
Group Ia	0.6 ± 0.1	3.1 ± 0.5
Group Ib	0.8 ± 0.2	3.1 ± 0.4
Group IIa	0.7 ± 0.1	3.6 ± 0.3
Group IIb	0.7 ± 0.1	4.1 ± 0.7
Group IIIa	0.5 ± 0.1	2.5 ± 0.2
Group IIIb	0.6 ± 0.1	3.5 ± 0.8

THE RADIOACTIVITY OF DNA IN LIVER AND EPIDERMIS AFTER ANTIGEN ADMINISTRATION AND LONG TIME EXPOSURE TO P32

Problem

Is the radioactivity relatively low in DNA of liver and epidermis 4 weeks after the administration of antigen and P^{32}?

Method

One month old male guinea pigs were used. The animals were divided into two groups and treated according to the schedule in Table 3.

[1] *Fraction I according to Lundin* containing inorganic P and organic P compounds readily soluble in trichloroacetic acid solution.

TABLE 3

	Number of animals	Average weight of group	Treatment on 1st day of exp	Time of P^{32} injection* after 1st day treatment
Group IVa	14	209 g	Primary Immunization §	2 days
Group IVb	16	199 g	1cc phys saline inj subcu	2 days

Note All injections were carried out at 9 am on the day specified Animals were sacrificed on the 27th day
* 0 5uC P^{32} as orthophosphate per g body weight injected subcutaneously
§ See above

The number of impulses per minute per mg DNAP in per cent of the number of impulses per minute per mg free P was calculated as well as the number of impulses per minute of all DNAP in the liver and epidermis in per cent of the number of impulses per minute per mg free P

RESULTS

Statistically significant differences were *not* found between the figures for DNA activity in liver and epidermis in the animals receiving typhi H antigen and those receiving saline (see Table 4)

TABLE 4

DNA Activity in Liver and Epidermis after Long-Time Exposure to P^{32}

	Impulses per mg DNAP × 100 / Impulses per mg free P		Impulses of all DNAP × 100 / Impulses per mg free P
	Liver	Epidermis	Liver
Group IVa	114 ± 17	102 ± 10	101 ± 17
Group IVb	184 ± 58	117 ± 21	132 ± 23

DISCUSSION

The absence of a difference between the activity of the liver DNA in antigen animals and controls after long-time exposure to P^{32} may be due to many reasons The described experiment deviates in many respects from the earlier experiment (*Fichtelius, Diderholm & Stillstrom* 1960), in which the antigen animals showed a lower activity of liver DNA The present experiment has been of value in one respect in showing that antigen administration had no disturbing effect on P^{32} incorporation into liver DNA A similar result was obtained by *Cooper* (1961) who found no difference in P^{32} incorporation into liver DNA after administration of human serum albumin to rats (if changes were present a slight increase was noted following administration of antigen) Thus the results of the earlier experiments (*Fichtelius* 1959 a and b, *Fichtelius, Diderholm & Stillstrom* 1960) can very well be explained in the following way Lymphocytes are normally destined for the liver, and

when antibody production is increased the lymphocytes are needed elsewhere and are drafted off from the liver or they never reach this organ

SUMMARY

The experiment is an attempt to confirm an earlier observation using another antigen and time schedule of a relatively low radioactivity in the liver DNA four days after the administration of P^{32} and antigen. The eventual disturbing influence of antigen administration on P^{32} incorporation into liver DNA was checked in experiments in which the animals were exposed to P^{32} for only 3 hours. Since some lymphocytes appear to be normally destined for the epidermis this organ was included in the investigation.

No differences were demonstrated in the activity of DNA of liver and epidermis between antigen animals and controls whether after short time long time exposure to P^{32}. The present experiment was of value in one respect in showing that antigen administration had no disturbing effect on P^{32} incorporation into liver DNA. This fact substantiates satisfactory the earlier conclusion. When antibody production is increased the lymphocytes normally destined for the liver are needed elsewhere and drafted off from the liver or never reach this organ.

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MULTIPLE DIFFUSE FIBROSARCOMATA OF THE BONES

By

ARNE RICHTER NIELSEN and HEMMING POULSEN

Received 22 xii 61

In 1944 *Paul E Steiner* described a case of multiple diffuse fibrosarcomas of the bones which, so far, represents the only one described in literature In 1959 a patient was admitted to the Sundby Hospital presenting, in many respects, the same clinical and pathological anatomical changes, because of the striking resemblance of the two cases we found both must belong to the same tumour type

The condition being a very rare occurrence and the present case adding new aspects to the picture as regards the nature of the tumour we thought it might be of general interest to give a report of the case

CASE REPORT

The patient is a 69-year-old housewife who at the age of 64 had been operated upon on account of [illegible]

[illegible] intumescense which was freely movable between the skin and the underlying tissue, this intumescense was located to the left of the trachea immediately above the jugulum, the act of swallowing made it move

The temperature was slightly elevated, the blood pressure was 190/100 mm The height of the patient 164 cm, her weight 44 7 kg

The remainder of the objective examination presented normal findings except for the knee and ankle joints of both legs which were sites of moderate polyarthritic changes

Roentgenographs on two occasions (May 20 and 27, 1961) of the left leg (cf [illegible] 1) disclosed a circular lesion medially and [illegible]

[illegible] six similar lesions were manifest in the

Horii I, Ito I & Sugimoto K The Management of Lymphocytes in the Liver and the Lymphocyte infiltration in the Hepatic Interstitum Acta Scholae Medicinalis Universitatis in Kioto Japonia 28 39 46 1950
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The patient is a 69 year old housewife who at the age of 64 had been [illegible] upon on [illegible]

[illegible]

prominence [illegible] cutaneous [illegible] n to [illegible] by a [illegible] the [illegible] to the left of the [illegible] above the jugulum, the act of swallowing made it move

The temperature was slightly elevated, the blood pressure was 190/100 mm The height of the patient 164 cm, her weight, 44 7 kg

The remainder of the objective examination presented normal findings except for the knee and ankle joints of both legs which were sites of moderate polyarthritic changes

Roentgenographs on two occasions (May 20 and 27, 1961) of the left leg (cf Fig 1) disclosed a circular lesion medially and [illegible] The les[illegible] f of the tibia. [illegible] lullary cavity, [illegible] well defined, [illegible] oth legs were [illegible] five or six similar lesions were manifest in the



Fig 1
Roentgenogram of the left leg, the lesion on the lower half of the tibia

Fig 2
Roentgenogram of the left thigh bone and the several osteolytic lesions

thigh of the left leg (cf Fig 2) two or three in the right All of the lesions involved the cortical bone in the left femoral body beginning just below the trochanter in the right just above the middle, the lesions were well defined

No rarefaction was observed in the pelvis the spine and the chest

The thyroid gland was enlarged and contained several calcifications whereas the renal and urinary tracts presented normal findings

Haemoglobin 83 69 per cent, erythrocyte count 4 31 mill per μl leucocytes 8 400 per μl eosinophile leucocytes 481 per μl thrombocytes 26 600 per μl, sedimentation rate 40 59 mm per hour serum phosphorus 4 7 mg per cent, serum calcium 9 8 mg per cent alkaline phosphatases 4 1 units per 100 ml acid phosphatases 0 40 units per 100 ml serum electrophoresis total protein 7 1 per cent including 3 46 of albumin and 3 64 of globulin Fractionated globulin increased $\alpha_1 + \alpha_2$ and γ normal β Urine A – S P Bence-Jones protein 0 metabolism 148 per cent Electrocardiogram normal findings

The bone changes of the patient were interpreted as metastases from an occult neoplasm and the patient was referred to the medical department for further examination At this stage the patient felt subjectively well apart from some fatigue loss of ... ight knee The case was considered to be o ... parathyroid—gland metastazising to the ... lished on the basis of findings of a solid ... left thyroid gland Otherwise the examir ... d roentgenographs disclosed continued growth of the tibial lesion on the left leg and again the patient was transferred to the surgical department where excision for the purpose of biopsy was made

At operation a lesion the size of a walnut was found and biopsies were taken from the solid as well as from the spongy bone tissue

Histological diagnosis Fibrosarcoma
Surgical measures being considered futile the patient was re transferred to the medical department to await admission to the Radiumstation, however she was rapidly sinking the temperature rising to 40° C the patient died 42 days after her first admission

AUTOPSY

Gross examination The patient is of slender frame, she is markedly emaciated and cachetic An arcuate thoracic kyphosis and a dextro-convex scoliosis are pronounced but the extremities are without deformities

Skin Pigmented areas are found, as already mentioned, on either leg The subcutaneous tissue is nodular to the touch, it has thickened and incision at this site reveals dispersed, small calcifications No tumour formation is present, in particular no fibromatoid formations, no symptoms of ichtyosis are demonstrated

Bones Incision medially on the left leg about 10 cm above the ankle penetrated into a soft, whitish fleshly tumour of about 6 × 7 × 8 cm invading a large lesion of the cortical bone of the tibia continuing into the medullary cavity The greater part of the cortical bone is destroyed by the tumour and laterally nothing remains except a papery coating of bone tissue There is neither haemorrhages nor any gross symptoms of necrosis, the tumour contains neither calcium nor bone tissue

Incisions into the right and left thigh bones reveal similar lesions which are filled up by the same type of tumour tissue as was noted in the tibia The thoracic and the lumbar vertebral bodies are sites of numerous, whitish and soft, infiltrations with diameters of 2 to 3 cm In this area the gross appearance reminds of multiple myelomas Other bone findings ore normal

The adrenals Both of the adrenal bodies are found slightly enlarged and centrally the tissue is of a greyish colour, rather soft the cortex is irregularly tapering

Peripheral lymph nodes, muscles, and breasts Normal findings

The thyroid gland On the cut surface are numerous colloid adenomata the right lobe remains unaffected but distally in the left lobe an adenoma of about 4 × 4 × 5 cm is demonstrated

The parathyroid gland No enlargement, no tumour formation

With the exception of some fibrous adhesions on the back of the right lung no anomalies are noted in the serous cavities

The lungs are sites of mild emphysematous changes including some stasis and edema in the basal areas No pneumonic changes, no metastases

Bronchi and mediastinal lymph nodes Normal findings

The heart Size of heart 11 × 8½ cm weight 390 g [illegible]

the arch to the

The pulmonary arteria, the venae cava superior and inferior, the portal vein: Normal findings

Oesophagus, stomach, and gastro-intestinal canal: Normal findings

The liver: Size: 22 × 13 × 5 cm; the shape, size, surface, cut surface, colour, structure are normal No tumour formation

The gallbladder contains 2 large cholesterol calculi of 2 and 4 cm, respectively, together with numerous smaller calculi The wall has thickened slightly Bile ducts: Normal findings

The spleen: Size: 12 × 6 × 4 cm; its structure is rather solid

The kidneys: Size of both kidneys: 12 × 6 × 3 cm The fibrous capsules are easily detachable, the surface is smooth In the cortical tissue of the left kidney a *solitary, circular, well-defined, rather solid, greyish white* tumour, fibroma? adenoma? metastasis? is found measuring 2 × 2 × 2 cm The tissue border is of normal size and pattern

The efferent urinary ducts. Normal findings

The reproductive organs A submucoid fibromyoma of 5 × 4 × 4 cm is found in the uterus together with several minor subserous fibromyomata The submucoid tissue of the large fibromyoma presents extensive calcification

The annexa Normal findings

The skull: Not autopsied

HISTO-PATHOLOGY

All sections originating from tumour formations in the right and left thigh bones, the left tibia, and several of the vertebral bodies (Figs 3, 4, and 5) reveal a presence of tumour tissue arranged in intertwining fasciculi of collagen fibrils interspersed with varying amounts of spindle cells Some areas are highly cellular, others are dominated by the numerous collagen fibrils

In some areas the elongated, rather slender, tumour cells are provided with highly uniform nuclei, the mitoses of which are scarce In other areas a pronounced polymorphism predominates and the hyperchromatism of the much larger nuclei is marked Here, the mitotic activity is more vivid and atypical forms are present There is a deficiency of cytoplasm, the outlines of the cells are blurred

The arrangement of cells differs in the various areas of the tumour Generally growth will occur in the intertwined strata and whorls, but occasionally the structure of the tissue is of a more uniform nature

Dispersed small necrotic areas and some minor infiltration of lymphocytes and plasma cells are found In addition to the necroses, occasional small haemorrhages may occur with few macrophages containing blood pigment Otherwise no pigmentation is demonstrated

The fibrils stain as collagen a m van Gieson-Hansen and Mallory, in the cellular areas some reticulin is seen using Wilders Silver Impregnation Method for Reticulum Fibers

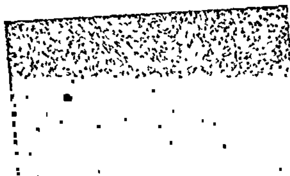


Fig 3
Photomicrograph of a characteristic tumour of the spine the structure of the tissue is marked by whorls (× 70)

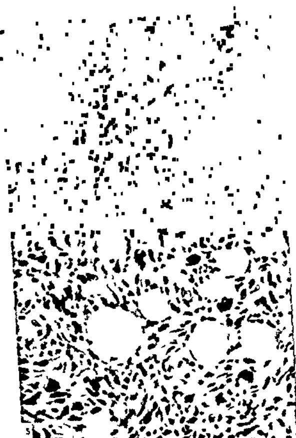


Fig 4
Tumour tissue from the left leg Remnants of the original fatty tissue are seen in the bone marrow (× 130)

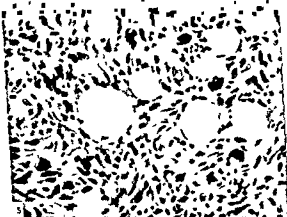


Fig 5
Higher magnification of Fig 4 (× 360)

The presence of myxomatous areas in the stroma was not demonstrated beyond doubt and staining using PAS and Alcian blue (*Eskelund* 1947) was negative A presence of cartilagenous tissue, osteoid tissue, and bone tissue could not be demonstrated

In sections from the long bones as well as from the vertebral bodies the medullary tissue was found to be the site of small infiltrations and all sections showed blurred outlines of the tumour tissue The bone tissue had been destroyed but no – or only scarce – periosteal reaction was found

Circular islets of tumour tissue were encountered in both of the adrenal bodies, identical to findings in the bones The adrenal tissue was compressed but otherwise normal

Sections from the thyroid gland demonstrate the presence of a colloid, calcified, adenoma, from a histological point of view the tumour demonstrated in the cortical tissue from the left kidney is an absolutely benign fibroma deviating significantly from the other mesodermal tumour formations

DISCUSSION

The condition here described is found to be a case of multiple fibrosarcomata in both of the thigh bones, in the left tibia, the spine, and both of the adrenal bodies, the lastmentioned being of a metastatic nature and very small Most of the bone tumours were arranged centrally in the bones without penetration of the cortical bones Only the tumour in the left tibia had exerted a destructive effect on the cortex and the periosteum but tumour growth was most marked centrally in the bone

A series including numerous sections from highly different sites of tumour formation in the bones of this patient presented fibroblastic tumour tissue without a tendency to formations of myxomatoid, chondromatoid or osteoid tissue, no deposits of calcium were found in the tumours

Geschickter 1932 and 1935, *Stout* 1948, etc maintained that actual fibrosarcomata originating from the endosteum are unknown, only those developing in the periosteum are encountered *Budd & McDonald* 1943, *Lichtenstein* 1959, etc differentiate between the central fibrosarcomata of the endosteum and sarcomata of the periosteum No doubt these authors are correct, in our opinion such purely fibroblastic tumour formation may occur centrally in the bones

If the case here discussed be compared with the one reported by *Steiner* (1944) lesions in the former case will be seen to occur in the thigh bones, the left tibia, and the spine in contrast to the latter in which *Steiner* found tumour formation in the left parietal bone, the sphenoid bone, the spine, the ribs, the sternum, and in the intermediate third of the thigh bones As opposed to *Steiner* who found widespread metastases to the viscera our case presented nothing but a small metastasis to the adrenal gland The more extensive bone destruction found in *Steiner*'s case may be ascribable to its more protacted course

Although the case reported by *Steiner* presented a more general

spread and a more pronounced destruction and collapsion of the bones it can hardly be denied that macroscopically as well as microscopically the tumours and their spread to the individual bones are so similar in these two cases as to suggest that they are of the same nature

Steiner voices the opinion that it may be a widespread sarcomatous change as known from *Paget s* disease However the only clue to this concept is the extensive deformities whereas the histological examination of bone specimens fail to lend support to such hypothesis because of the absence of features characteristic of that disease

The present case demonstrated no symptoms of a Paget s disease which consequently can be precluded as etiologic factor

Whether the tumour is of a solitary or multi centric origin cannot be elucidated on the basis of findings from the present case because in contrast to *Steiner* we have no roentgenograms of bones demonstrating the status before the tumours became visible

In the case reported by *Steiner* ichthyosis was widespread as opposed to our case where no such symptoms were manifest for which reason it is considered highly improbable that there should be any etiological interrelation between this disorder and the malignant disease

Moreover *Steiner* has taken into consideration whether the finding in his case of the numerous naevi in some way might explain its relation to the malignant disease but neither in his case nor in the present one were any suspicious naevi visible and the histological findings provides no basis on which to suggest such diagnosis viz malignant melanoma

The localized subcutaneous calcinosis involving both crura escapes a definite explanation It can hardly be ascribable to a localized tumour effect because if so the calcinosis should have been leftsided exclusively Besides it is open to discussion whether a connection may be established between the calcinosis and the multiple tumours and it should be noted *Steiner* describes no presence of a calcinosis Also the literature fails to provide details as to a probable interrelation of such calcinosis and the mesodermal tumours

Whereas *Steiner* does not feel convinced about the neurogenic origin of the sarcoma reported by him we find many features supporting such opinion *e g* the intertwining bundles the fasciculi and the whorls If correct it might be a case of malignant transformation in a single neurofibroma involving the bones upon which the other tumours are to be considered as metastases but the multiple central fibrosarcomata involving the bones may also represent the malignant counterpart to the multiple osteogenic neurofibromata (*Miller et al* 1957 *Hulshoff* 1960)

SUMMARY

A case o'
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occurrence of a malignant transformation in the multiple osteogenic neurofibromas is suggested

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FROM THE BISPEBJERG HOSPITAL SURGICAL DEPARTMENT D (HEAD PROF J C CHRISTOFFERSEN) AND THE BIOLOGICAL INSTITUTE OF THE CARLSBERG FOUNDATION COPENHAGEN DENMARK

THE FIBRINOLYTIC ACTIVITY OF TUMORS OF THE KIDNEY AND BLADDER

By

AA LADEHOFF

Received 22 xii 61

The increased fibrinolytic activity which sometimes is found in metastatic cancer of the prostate (*Tagnon et al* 1953 *Dolaz et al* 1955) may direct the attention to a possible relationship between the fibrinolytic activity of neoplastic tissues and the spread and infiltrative growth of tumors

Clifton & Grossi (1955), using the method of *Permin* (1950), studied the fibrinolytic activity of more than 200 tumors of various locations and degrees of malignancy, but because of the semiquantitative estimation method a more exact comparison between the activity of normal and neoplastic tissues was not possible With the quantitative method of *Astrup & Albrechtsen* (1957) the plasminogen activator activity of normal (*Rasmussen & Albrechtsen* 1960) and hyperplastic prostates (*Ladehoff & Rasmussen* 1961) was investigated

Kidney carcinomas have a tendency to early vascular spread and malignant bladder tumors rapidly infiltrate the bladder wall This paper presents an investigation of the content of plasminogen activator in tumors of the kidney and bladder, normal tissues of the human urinary tract having been studied previously (*Ladehoff* 1960) It is attempted to correlate the fibrinolytic activity of the bladder tumors with their histological types using the classification of the Institute of Urology London (5)

MATERIAL AND METHODS

The material comprises 17 tumors of the kidney and 61 of the bladder Tissue samples were obtained from fresh operation specimens In the renal cases samples were taken from macroscopically different areas of the tumor when such were found and for comparison from normal cortical and medullary kidney tissue as well Normal bladder tissue could be obtained only in 4 cases of hemi or total cystectomy but not in transvesical or transurethral localized resections by which the majority of the bladder tumors were treated

The samples were investigated immediately or after storage for a few days at 20° C. The fibrinolytic activity was estimated by the method of *Astrup & Al*

This investigation was supported by grants to Dr *T Astrup* from the *Josiah Macy Jr Foundation* New York

occurrence of a malignant transformation in the multiple osteogenic neurofibromas is suggested

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TABLE 2

35 Bladder Tumors with a Slight or Moderate Content of Plasminogen Activator

No	Sex Age	Activity (units/g)	Site of tumor: High	Site of tumor: Low	Benign papilloma	Transitional cell carcinoma: Diff	Transitional cell carcinoma: Undiff	Solid	Papillary
1	M/57	0		X			X	X	
2	M/50	0	X			X		X	
3	M/65	+	X		X				
4	M/62	24		X			X		X
5	M/56	34	X			X			X
6	M/55	34		X	X				
7	M/78	43	X				X	X	
8	M/62	50		X	X				
9	F/41	56	X					X	
10	M/67	68	X				X	X	
11	F/69	72		X			X	X	
12	M/59	80		X	X				
13	M/67	92		X		X			X
14	M/54	121	X				X	X	
15	F/58	135	X			X			X
16	M/74	135	X				X		X
17	F/56	139		X	X				
18	M/61	147	X			X			
19	F/73	153		X				X	
20	M/70	165	X				X	X	
21	F/60	170	X			X			X
22	M/61	179	X			X		X	
23	F/70	189		X			X	X	
24	F/57	207	X				X	X	
25	F/56	210		X		X			X
26	M/71	240	X				X		X
27	M/73	252	X				X	X	
28	M/62	252	X			X		X	
29	M/64	266		X		X			X
30	M/66	273		X		X			X
31	F/81	274	X			X			X
32	M/56	300		X	X				
33	M/67	304	X				X	X	
34	M/52	340	X				X	X	
35	M/63	370		X		X			X
						X			X

Bladder Tumors

The 61 tumors may be divided into 4 groups on the basis of the concentration of tissue activator, expressed in units per gram fresh tissue 1) 0–100, 2) 100–400, 3) 400–900 and 4) above 900 units/g. The first two groups, with a slight or moderate activity, are placed together in table 2, and those with a high or extremely high activator content appear in Table 3.

The bladder is of dual origin, the smaller basal part being derived from the mesoderm and the rest from the endoderm. Since this dual evolution might be reflected in the fibrinolytic activity of neoplastic

brechtsen (2), the concentration of plasminogen activator being expressed in units of a standard preparation per gram fresh tissue

Histologically the *kidney tumors* were all classified as carcinomas of the hypernephroma type. Using the histological classification of the Institute of Urology (5) the *bladder tumors* were divided into the following groups the three first of which comprised the majority of the cases (1) benign papilloma (2) differentiated transitional cell carcinoma (papillary or solid), (3) undifferentiated or anaplastic transitional cell carcinoma (papillary or solid) (4) squamous cell carcinoma and (5) adenocarcinoma

RESULTS

Kidney Carcinomas

The content of plasminogen activator in the 17 hypernephromas appears from table 1. Values obtained from normal renal tissue from the same patient are recorded for comparison

TABLE 1

Content of Plasminogen Activator in 17 Hypernephromas Compared with the Activity of Normal Kidney Tissue

No	Tumor tissue		Normal kidney tissue	
	1)	2)	Cortex	Medulla
1	0	—	171	—
2	0	78	88	405
3	0	288	162	162
4	0	1440	432	
5	+	40	25	40
6	11	0	108	798
7	34	58	50	252
8	50	—	64	839
9	63	378	76	252
10	183	—	57	88
11	252	145	340	—
12	252	—	39	315
13	401	—	155	401
14	529	0	107	259
15	720	0	192	528
16	836	11	513	1026
17	—	468	570	935

The figures represent activator units per gram fresh tissue
Tumor tissue 1) = soft more or less necrotic part
2) = solid fibrous like part

The activity was found to vary as much as the gross and microscopic appearance of this tumor type. As an example the activator content might range from 0 to 1440 units per gram tissue in macroscopically different areas of the same tumor. Large individual variations of activity were also demonstrated. In only a few cases the activity of the tumor was higher than the activity of the corresponding normal kidney tissue. The medulla of the latter was usually more active than the cortex

In 4 cases was it possible to compare the activator content of malignant and normal tissue from the same bladder (Table 4). In three of these cases the tumor tissue was less active than the corresponding normal mucous membrane and in one the activities were equal.

TABLE 4
Fibrinolytic Activity of Malignant and Normal Bladder Tissue from 4 Cases of Hemi- or Total Cystectomy

No.	Tumor	Normal bladder tissue	
		Mucosa	Muscularis
4	24	472	189
10	68	329	
14	121	264	92
49	1125	1140	636

DISCUSSION

The cellular origin and localization of the tissue activator have not yet been established. Using frozen tissue sections *Todd* (1959) adapted the fibrin plate technique for histological use. The foci of fibrinolysis could be seen in relation to certain structures, chiefly veins and venules. Only normal tissue was investigated.

In Glisson's capsule of the human liver, which contains numerous mast cells, *Ende & Auditore* (7) found moderate amounts of tissue activator, while the liver usually had no or slight activity. A high fibrinolytic activity was observed in mast cell tumors of the dog and in a study of the intracellular distribution the greatest concentration of tissue activator in a mast cell sarcoma was found in the microsomal fraction (6, 7).

In case of a possible relation between the fibrinolytic activity of neoplastic tissues and their spread an increase in activity with *increasing* malignancy could be assumed to favour *the growth of* the primary tumor and disseminated tumor cells by the lysis of fibrin produced as part of defence reaction of the organism. The opposite view, that fibrinolysis might prevent the lodging and growth of the tumor cells, has been suggested by *Grossi et al.* (1960) who observed an effect of human fibrinolysin on pulmonary metastases in animal *experiments*.

So far the only systematic study of the *fibrinolytic* activity of human tumors has been published by *Clifton & Grossi* (3), who tested more than two hundred different *tumors*. Because of the semiquantitative estimation method which was *used*, a conclusive evaluation of the results, however, was not possible.

When considering the results of the *present* estimations of the concentration of plasminogen activator in *tumors of the* human kidney and bladder it may be mentioned that in normal tissues of the urinary

bladder tissue the tumors are also grouped according to their site those localized within the trigonum or in close relation to the ureteral openings are characterized as situated low and those localized above as high

TABLE 3

27 Bladder Tumors with a High or Extremely High Content of Plasminogen Activator

No	Sex Age	Activity (units/g)	Site of tumor High	Site of tumor Low	Benign papilloma	Transitional cell carcinoma Diff	Transitional cell carcinoma Undiff	Solid	Papillary
36	M/78	456		×			×		×
37	M/66	479		×			×	×	
38	M/68	483	×			×			×
39	M/85	501	×			×			×
40	M/66	504		×		×			×
41	F/55	547		×	×				
42	F/75	567		×			×		×
43	F/48	570	×			×			×
44	M/57	579	×				×	×	
45	F/62	689	×				×	×	
46	M/53	700	×				×	×	
47	F/63	912		×	×				
48	M/63	1055		×		×			×
49	M/67	1125		×		×			×
50	M/74	1500		×		×			×
51	M/78	1550		×			×		×
52	F/52	1603		×			×	×	
53	M/20	1638		×		×			×
54	F/60	1688		×	×				
55	M/79	1787		×		×		×	
56	M/54	1960	×			×			×
57	F/39	2062		×		×			×
58	M/90	2736		×	×				
59	F/54	2835	×				×	×	
60	F/58	3876	×			×			×
61	F/65	4332		×			×	×	

Great variations of the activator content were found and obviously they were independent of the histological type as well as of the site of the tumors. Very low and very high activities were demonstrated in benign papillomas as well as in differentiated and undifferentiated carcinomas whether the tumor site was high or low.

Two cases were inactive and in one the activity was too small for a quantitative estimation. An amount of tissue activator of less than 100 units/g occurred in 13 of 61 tumors (21 per cent). In 22 tumors (36 per cent) activities of 100–400 units were found. A high activator content of 400–900 units was demonstrated in 11 cases (18 per cent) and the group of extremely high activity (above 900 units) included 15 cases (25 per cent).

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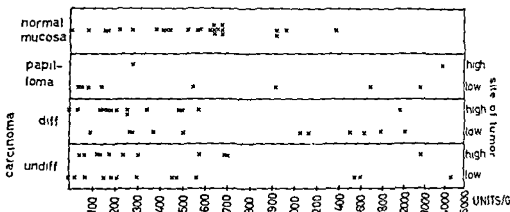


Fig 1

Tissue activator content of 61 bladder tumors in relation to histological diagnosis and site in the bladder — For comparison the mucous membrane activities of 25 normal bladders are recorded (*Ladehoff* 1960)

tract (*Ladehoff* 1960) a marked difference was observed between the activity of kidney parenchyma and the urine conducting tissues, the latter being most active Considerable variations, however, were found within the same tissue groups from different persons, as is the case in other human organs (*Albrechtsen* 1958, *Ende & Auditore* 1961), to some extent the same conditions are found in tumors of the urinary tract

In kidney carcinomas, which have a strong tendency of early and massive spread by the blood stream (*McDonald & Priestly* 1943), the content of plasminogen activator was on the average slight or moderate and usually not higher than in the corresponding normal kidney tissue

Malignant bladder tumors rapidly invade the bladder wall, they spread mainly by the lymphatics and haematogenous metastases appear rather late even if tumor cells have been demonstrated in the blood in relation to operative management (*Jonasson* 1961) A little more than half of the present material of bladder tumors had a slight or moderate activator content while the activity of the rest was high or extremely high Among the latter several cases appeared with the greatest concentrations of tissue activator which up to now have been found in this laboratory The mucosal activities of normal bladders also showed great individual variations, but the highest activities were lower than those found in the most active tumors (Fig 1)

In so far as it can be evaluated from the site of the tumor no significant relationship could certainly be found between activity and the dual embryological origin of the bladder However, 11 of the 15 tumors with an extremely high activator content were situated within the basal part of the bladder, which originates from the mesoderm (Fig 1)

The great variations in activity without significant correlation with histological malignancy may be explained in two ways either that the tumor activity depends upon the activity of the tissue of origin, which

varies individually, or the fact that the morphological histological grading yields no information of possible differences in enzymatic activity of tissues of the same histological picture, but of varied biological malignancy

The mono symptomatic haematuria of variable strength and duration which is the predominant symptom of tumors in the urinary tract may be related to the tissue activator activity, as fibrinolysis may influence local haemostasis

SUMMARY

The concentration of plasminogen activator was estimated in 17 hypernephromas and in 61 bladder tumors of different degrees of malignancy

The activator content of the kidney carcinomas varied individually as well as in macroscopically different areas of the same tumor, but was usually not greater than in the corresponding normal kidney tissue

In the bladder tumors the activator activity ranged from slight to extremely high without significant correlation with the histological degree of malignancy In 3 of 4 cases of cystectomy the activity of the tumor was less than in the corresponding normal bladder mucosa and in 1 the activities were equal

It may be assumed that the tumor activity depends upon the activity of the tissue of origin, which varies individually, or that tumors of the same histological type differ as to enzymatic activity and thus also as to biological malignancy

The haematuria in tumors of the urinary tract may be related to the tissue activator activity as fibrinolysis may influence local haemostasis

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FROM CANCERREGISTERET AND DEPARTMENT OF PATHOLOGY, FINSEN INSTITUTE COPENHAGEN, DIRECTOR JOHANNES CLEMMESEN, M.D.

STUDIES ON THE NEWT TEST FOR CARCINOGENICITY

2. *Tributyrin and Propylene Glycol as Solvents*

By

E. ARFFMANN

Received 12.i.62

Previous experiments (*Arffmann & Christensen* 1961) have confirmed the findings of *Neukomm* (1957) that the three potent carcinogens, benzo[a]pyrene, dibenz[a,h]anthracene, and 3-methylcholanthrene regularly induce epithelial hyperplasia and downgrowth on subcutaneous injection in newts (Triton cristatus). Only one out of 115 controls injected with pure oil solvent, mostly peanut oil, showed a clearly positive result. Injections with ordinary oils and fats, however, may occasionally give rise to a few tumors in rodents (*Arffmann* 1960), perhaps due to the somewhat varying composition of vegetable and animal lipids. Planning a series of experiments on the specificity of the newt test, we therefore found it advisable to examine the effect of some chemically well defined lipophilic solvents on the newt. To this end tributyrin and propylene glycol were chosen, both being easily available.

Tributyrin is an ester of glycerol and butyric acid. It has not been widely used as a solvent, but in the few experiments on injection of tributyrin in rats (*Davis* 1930), and mice (*Lettre & Fernholz* 1940), local lesions were not observed.

Propylene glycol is a divalent alcohol. This solvent has served as a vehicle to a variety of compounds and in experiments on several species of animals (*Hartwell* 1951 and 1957). *Bradbury et al.* (*1941*) found no difference in carcinogenic action on mice whether 9,10-dimethyl-1,2-benzanthracene and its 5-n-propyl derivative were applied subcutaneously in sesame oil or propylene glycol. While sesame oil persists for months at the site of injection, propylene glycol is absorbed, leaving the carcinogens as a crystalline material in the subcutaneous tissue. Most of the hydrocarbons used were rather insoluble in propylene glycol and hence they were injected as fine suspensions in this solvent.

In experiments on the carcinogenic activity of 2-acetaminofluorene and related compounds *Wilson et al.* (1947) found indications that propylene glycol may not be quite indifferent. Among 21 control and experimental rats receiving propylene glycol subcutaneously, 6 developed leukemia in 202 to 600 days (*Wilson et al.* 1947 b). Leukemia

was not observed in non-experimental rats nor in rats in other experiments. Whether propylene glycol was the causative factor could not be determined on the basis of the recorded, quantitatively rather sparse results.

Hartwell (1951 and 1957) surveys a rather large number of other injection experiments using propylene glycol as solvent, among these studies on steroid hormones. The animals were mice, rats, guinea pigs, rabbits and dogs. *Boyland & Sargent* (1951) in one experiment used mustard gas in propylene glycol, studying the local greying of hair in mice. Most of these experiments are, however, of no interest in connection with the present work.

TECHNIQUE

The experimental procedures were principally those earlier described (*Arffmann & Christensen* 1961). In order to avoid misinterpretation of unspecific epithelial lesions at the site of needle puncture this place, marked by a suture, was omitted at histological examination. The optimal period for estimation of the reaction being the second and third weeks, the last tails were amputated on the 20th day.

Tributyrin was obtained from The British Drug Houses Ltd., England (Specifications: Assay (saponification): not less than 98 per cent. Wt. per ml at 20° C 1.030 to 1.040 g. Refractive index (n_D) 1.436 to 1.437. Free acid: not more than 5 ml N/1 per 100 g). Benzo[a]pyrene was easily solved in the tributyrin at 0.1 per cent (w/v) by simple stirring for about 10 minutes without the use of heat. This preparation was injected into the tail of 9 Triton cristatus, 9 salamander controls receiving tributyrin only.

Benzpyrene and especially methylcholanthrene were weakly soluble in propylene glycol. It was intended to examine the effect of stronger solutions of the carcinogenic hydrocarbons on the newt, but it proved impossible to obtain a clear solution of methylcholanthrene at the concentrations of 1 mg, 2 mg or 5 mg per ml of solvent in spite of vigorous stirring at 100° C. The same applied to benzpyrene at 5 mg to 1 ml of propylene glycol. As the other experimental results in these studies are based on the use of clear solutions, it was considered inadequate to use propylene glycol as solvent in experiments with a comparing aim.

In agreement with earlier experience benzpyrene appeared more easily soluble than methylcholanthrene. After intermittent stirring at 100° C for 20 to 30 minutes
ar. At 2 mg per ml rapid secondary
was injected into 4 animals, while
on after heating at that moment
solution was injected into 9 salamanders, while 9 controls received only propylene glycol (heated at 100° C for 10 minutes).

RESULTS

Tributyrin as solvent. In most of the surviving controls and experimental animals tributyrin provoked a large ulceration at the site of injection. This lesion was maximal about the ninth day, at which time 4 controls and 5 benzpyrene injected salamanders had to be killed because of severe changes of the tail. The proximal part was the seat of an irregular and deep ulceration occupying the whole area of injection and extending to the anus, sometimes even involving the latter and spreading to the left side of the tail. Distally to the ulceration the tail was totally necrotic and often partly or wholly thrown off. The zone of demarcation was located at the site of injection just proximal to th







Fig 1 Ulceration with severe inflammation on 9th day after tributyrin injection Below regenerating epithelium is seen on the surface

Fig 2 Large ulcerative defect on 9th day after injection of benzpyrene in tributyrin Below the anus and to the left of this hyperplasia of epidermis

Fig 3 Moderate epidermal hyperplasia at the edge of an ulcer on the 9th day after tributyrin injection

at the distal edge of the anus so that the whole area of injection was necrotic One control animal dying on the fifth day showed a large ulceration

Histological examination of the described lesions did not reveal differences between newts injected with the carcinogen, and controls In the ulcerated area extensive destruction of all tissues was manifest reaching the spine and involving this where necrosis began Edema small bleedings and a heavy infiltration with leucocytes, including many of the polymorphonuclear variety, were signs of severe inflammation (Fig 1) The large irregular defect (Fig 2) was partly covered by rapidly regenerating epidermis, which grew from the edges This epithelium often showed moderate signs of hyperplasia (Fig 3) and small downgrowths might be seen, but these changes were quite overshadowed by the ulcerative and necrotic inflammation The epithelium was growing on deposited fibrin with no pigmented layer below

Distally to the ulceration the tail was necrotic, sometimes leaving shadows of nuclei, especially in epithelium and connective tissue

Two controls and two experimental animals escaped the severe necrotic lesions and were examined on the 15 and 21 day after injection The results were (+) (slight localized hyperplasia) and ± after benzpyrene, while both of the controls were negative, one with an ulceration covered by non-hyperplastic epithelium It is difficult to see a decisive result in this

Three animals died on the first day after injection, and one benzpyrene-treated salamander died on the 9th day with no epithelial reaction

Propylene glycol as solvent On macroscopic examination two controls and two experimental animals showed flat ulcers at the site of injection The rest of the 31 salamanders were without gross changes

TABLE 1

Experiment with Benzpyrene in Propylene Glycol

Substance	Propylene glycol	Benzpyrene 0 1 per cent in propylene glycol	Benzpyrene 0 2 per cent in propylene glycol
Number of animals (Triton cristatus)	9	9	13
Epidermal reaction on			
1 3 day		+ —	—
10 11 day	(+) —		— (+) —*
15 day			
20 day		(+) +	± +
Animals died during experiment	2 (1 included)	0	2 (included)

* Died on 17th day



Fig. 4 Edema and slight inflammatory infiltration on the 11th day after propylene glycol injection

Fig. 5 Strong inflammatory reaction on the 15th day after injection of benzpyrene 0.2 per cent in propylene glycol. No epidermal hyperplasia

Fig. 6 Epithelial hyperplasia and beginning downgrowth on the 20th day after injection of benzpyrene 0.2 per cent in propylene glycol

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The microscopic results are summarized in the table. The slight difference between controls and carcinogen-treated newts indicates a positive reaction on benzpyrene, but both the 0.1 and 0.2 per cent solutions had the same weak effect. At the higher concentration the positive responses were found among 9 animals injected with the cloudy solution.

The unspecific reactions were local edema splitting up the normal tissues and accompanied by a moderate, sometimes strong inflammatory infiltration (Fig. 4 and 5). In cases of ulceration this was covered by regenerating epithelium, hyperplastic with slight, doubtful signs of infiltration in one control animal, but showing both hyperplasia and downgrowth in the two experimental animals (Fig. 6).

DISCUSSION

In contrast to injection experiments on rodents (*Davis* 1930, *Lettre & Fernholz* 1940) feeding experiments with tributyrin are in accordance with the present results on injection of the compound in newts. *Eckstein's* (1929) rats simply refused diets containing tributyrin on account of its intense bitterness. *Takahashi* (1926) found it strongly noxious and 2 of his 4 rats on the 5 and 10 per cent tributyrin diets died in about 20 days. *Davis* (1930) who fed tributyrin by pipette to chickens observed inflammatory lesions in the gastointestinal tract within 48–72 hours. In some cases apparent destruction of the mucosa was seen in the fore part of the small intestine. A toxic effect in rats is recorded also in this work. Most interesting are the results of *Salmon and Copeland* (1949) who fed rats on synthetic diets with 15 and 25 per cent of tributyrin. Sixty-six rats came to autopsy after periods varying from 3 to 35 weeks. In all of these animals the entire mucosa of the fore stomach presented a mass of papillomas. Occasional ulcerations were seen and in numerous cases histological examination revealed epithelial penetration of muscularis mucosae. Ten rats showed lesions in the glandular stomach, and in 5 of these proliferating gland elements penetrated the submucosa infiltrating extensively the external muscle layers. The lesions were not classified as definitely precancerous. They were attributed to a direct irritative effect of tributyrin.

It is well-known that the toxicity of a substance may cause a local tissue necrosis masking its carcinogenic effect (*Bradbury et al* 1941). In the present experiments the solvent (tributyrin) showed a toxicity of this degree and so masks the specific action of a solved carcinogenic substance. Reduction in dose of applied tributyrin is not possible if a minimal subcutaneous deposit is to be secured. Hence tributyrin is *not* suited as a solvent in the newt test.

Is tributyrin carcinogenic? The moderate epithelial reaction seen especially at the edges of the tributyrin-induced ulcerations is morphologically similar to findings in positive cases. Yet the picture is diffe-

rent, here dominated by the overwhelming unspecific ulcerative lesion, to which the weak epithelial reaction is out of proportion Carcinogens, on the other hand, induce a vigorous epithelial response which in most cases dominates the histological appearance whether or not there is an ulceration *Salmon & Copeland*'s results do not definitely deny a certain carcinogenic effect of tributyrin, and this may in our experiments be masked by the toxic effect, but in view of the high regenerative potency of the epithelium of the newt, the moderate reaction on the severe destruction of tissue seems a natural consequence and so most probably is unspecific

In prolonged feeding experiments on several species of animals propylene glycol was shown to be practically devoid of demonstrable toxicity (*Hanzlik et al* 1939, *Kesten et al* 1939) The experiments of *Morris et al* (1942) covered more than 2 years, but no case of tumor nor of leukemia was noted

In accordance with these studies the author's experiments showed no signs of a local toxic effect of propylene glycol, apart from a flat ulceration in one, negative control

This solvent, however, was not able to produce a clear difference between controls and salamanders injected with the carcinogen Furthermore, the examined hydrocarbons were only weakly soluble in propylene glycol, a severe drawback impeding the attainment of comparable results By way of conclusion propylene glycol could not be considered a suitable solvent for the newt test

Tricaprylin was not tested The cost is high, it is difficult to obtain, and therefore unsuitable for routine experiments Furthermore, *Steiner & Falk* (1951) obtained 3 sarcomas in 233 control mice, injected with tricaprylin They conclude that it is not an inert solvent, since it has a low degree of carcinogenicity comparable to that of some other oily vehicles

On the background of the results here reported it was decided to continue with refined peanut oil and soyabean oil as vehicles in the future studies on the newt test Earlier results (*Arffmann & Christensen* 1961) justified this

SUMMARY

Two chemically well-defined lipophilic solvents, tributyrin and propylene glycol were tested on Triton cristatus Tributyrin had a severe toxic effect, leading to deep ulceration at the site of injection and extensive necrosis of the tail Propylene glycol prooved to be a poor solvent for the hydrocarbons, and in spite of a few positive reactions on the carcinogen results on injection did not yield a marked difference between controls and salamanders injected with benzpyrene in a 0 1 or 0 2 per cent solution It is concluded that tributyrin and propylene glycol are not suitable solvents in the newt test

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The author is indebted to Professor E Boyland, The Chester Beatty Institute, London, for valuable advice concerning the solvents

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FROM THE UNIVERSITY OF BERGEN, SCHOOL OF MEDICINE, THE GADE INSTITUTE, DEPARTMENT OF MICROBIOLOGY, BERGEN, NORWAY

IMMUNOCHEMICAL STUDIES ON POLYSACCHARIDE A OF STAPHYLOCOCCUS AUREUS

3. *Some Chemical and Physicochemical Properties*

By

GUNNAR HAUKENES

Received 25.xii.61

The purification of polysaccharide A from two staphylococcal strains by ion exchange chromatography has been described in two preceding papers (15, 16). Strain Wood 46 was found to give a relatively large yield, and a polysaccharide A preparation of an apparently high degree of purity was obtained.

This paper presents some chemical and physicochemical properties of polysaccharide A Wood 46. Comparison is made with polysaccharide [illegible]

[illegible] results have been reviewed by *Oeding* (29), and will not be discussed here as it is uncertain whether polysaccharide A was the active principle of these substances.

MATERIALS

Preparation 1. Crude polysaccharide 1503 obtained from extracts of crushed bacteria by alcohol precipitation after removal of acid and alkali precipitates (15, 17).

Preparation 2. Prepared from the crude polysaccharide above by chromatography on DEAE cellulose columns (15).

Preparation 3. Prepared in the same way as preparation 2 above, but from another batch of crude polysaccharide.

Preparation 4. Prepared from a third batch of crude polysaccharide 1503 and purified by chromatography on DEAE cellulose and Dowex 1 columns (16). The material was contaminated with small amounts of *Jensen*'s antigen A (21).

Preparation 5. Polysaccharide A Wood 46 purified [illegible]

De[illegible]

be[illegible]

of[illegible]

METHODS AND METHODOLOGICAL CONSIDERATIONS

[illegible]

capillary space and thereafter allowed to flow back by its own weight The time taken by the meniscus to flow from the mark above to the mark below the capillary space was recorded with a stopwatch, and was about 92 seconds for distilled water at 21° C Four to 6 determinations were made

The water bath was not equipped with a thermostat and the temperature was therefore recorded for each determination It usually remained constant during the first two or three determinations, whereupon a rise of 0 1° C was observed The determinations were carried out within ± 0 05° C, and the time values usually agreed within 0 1 second According to *Kabat & Mayer* (24) 4 determinations should agree within 0 4 second and the temperature of the water bath should be maintained within ± 0 05° C Our determinations accordingly fulfilled these demands

The relative viscosity can be calculated from the equation

$$\text{Relative viscosity} = \frac{t_{\text{solution}}}{t_{\text{solvent}}} \quad \frac{d_{\text{solvent}}}{d_{\text{solution}}} \quad (24)$$

where t is the time recorded and d the density The density of a 0 5 per cent polysaccharide A solution has not been estimated It is however, less than 1 005 and is therefore of little consequence for the values obtained

Optical rotation was measured with a Zeiss Circle Polarimeter 0 01° with a sodium lamp and adjustable half shade angle The smallest possible half shade angle was selected to obtain the highest degree of sensitivity Diminuation of the angle however reduced the brightness of the setting fields Readings were therefore made in a dark room after the eyes had been adapted to the dark for at least 15 minutes Four observations were made from each direction on the scale The specific optical rotation was calculated from the formula

$$[\alpha]_{D}^{t^\circ} = \frac{\alpha \ 100}{l \ c},$$

which expresses the specific rotation at t° C with sodium light (D), when l is the length of the tube in decimeter c the concentration in grams per 100 ml and α the angle recorded

Biuret test The technique was the same as described in (17) The sensitivity of the test was examined with various concentrations of crystalline egg albumin A positive reaction was obtained with a 0 05 per cent solution and a negative or doubtful reaction with a 0 03 per cent solution

Molisch test The test was carried out as described in (17) The sensitivity of the test for glucose was examined A positive reaction was obtained with 8 μg per ml and a very weak reaction with 4 μg per ml Glucuronic acid gave a negative reaction and glucosamine produced a yellow to orange colour quite different from the intense violet or purple colour given by most carbohydrates

Bial's test was used for pentoses (2) The lowest concentration of arabinose *yielding a positive reaction was 2 μg per ml Boiling for* 5 *minutes* was used as a routine Glucuronic acid produced the same beautiful green colour as pentoses Positive reactions therefore require additional tests *e g* the naphtoresorcinol or the aniline test to differentiate between pentoses and uronic acids

Dische's diphenylamine test (7) was used for deoxypentoses Positive reactions need additional spectrophotometric testing since violet colours are given by *e g* fructose (34) and sialic acid (31) the latter having a maximum at 530 mμ On the other hand negative reactions do not exclude deoxyribose since only deoxyribose connected to purines reacts in Dische's test (26) Deoxyribose linked to pyrimidines is not released under the conditions employed and supplementary examinations of the ultraviolet spectrum and paper chromatography may be required

Nitrogen determination was carried out by the micro Kjeldahl method as described in (24)

Phosphorus estimation Total phosphorus was estimated by the method of *Fiske & Subbaro* ... ad of nitric acid After digestion ... meta and pyrophosphates to ... stimated by the same method but without digestion Acid was then added to the reagents to get the same concentration of acid as in the digest This method has been studied extensively by

Gierlsen (13) He examined the colour development with several standards at intervals of 3 minutes, and found that the colour was fully developed within 30 minutes and thereafter remained constant Readings were therefore made after 30 minutes

changed up to 6N for 6 hours Slightly lower values were obtained after 24 hours with 6N hydrochloric acid

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TABLE 1

Colour Intensities at 530 and 505 mμ given by Glucosamine Standards and a Hydrolyzed Sample of Polysaccharide A Wood 46 by the Method of Rondle & Morgan (30).

Glucosamine standards and a hydrolysate of polysacch. A		$E_{530\,m\mu} \times 1000$ Readings after hours			$E_{505\,m\mu} \times 1000$ Readings after hours		
		0	1½	24	0	1½	24
	25 μg	148 147	150 145	145 141	96 96	100 99	110 110
	50 μg	301 296	290 284	267 258	204 198	196 191	204 198
	75 μg	439 437	414 415	371 369	294 295	281 283	282 281
	100 μg	548 560	525 527	456 458	371 378	361 361	352 355
	150 μg	740 763	684 711	578 600	515 523	477 491	471 478
Polys. A W 46 hydrolysate	250 μg	351 349	340 336	285 289	248 252	250 252	249 252

Reducing sugars present after hydrolysis were determined by the Hagedorn Jensen method as modified by *Hanes* (14) A calibration curve was plotted for each determination using glucose standards covering the range from 0.2 to 4 mg The standards were not heated but *sodium chloride* was added to the same concentration as in the neutralized hydrolysates

Fu
r

mμ. One hundred μg of glucosamine gave an extinction of about 0.55 with a 1.0 cm cell and a final volume of 10 ml. The extinction values given by 20 to 100 μg of glucosamine usually showed a linear relationship to the weight of sugar, the value for 100 μg sometimes being a little below the straight line. For quantities of glucosamine over 100 μg Beer's law was not strictly applicable. The wavelength of maximum absorption shifted from 530 mμ to 510 mμ in the course of 5 days, the drift being faster with greater amounts of glucosamine.

The stability of the colour was examined with five duplicate standards of glucosamine and a hydrolyzed sample of polysaccharide A Wood 46. The colour given by 25 μg remained almost unchanged for 24 hours at 530 mμ and showed some increase at 505 mμ (Table 1). The extinctions of the other standards decreased during the observation time more pronouncedly with the higher values. The hydrolyzed sample behaved like the glucosamine standards. As a routine, therefore, the spectrophotometric recording is taken immediately after finishing the procedures and a second reading after 24 hours. The latter reading has been performed as muramic acid shows a maximum at 505 mμ with considerably increased values after 24 hours (5). The quantities of material in the unknowns are regulated to give hexosamine values within 25 to 75 μg of glucosamine.

RESULTS

General properties. Polysaccharide A Wood 46 was a white amorphous, rather hygroscopic powder. It dissolved readily in water yielding a clear colourless solution even in a concentration of 5 g per 100 ml.

The material was not precipitated by trichloroacetic acid, mineral acids or alkali, and could not be salted out with ammonium sulphate or sodium chloride. At pH 5.2 and in the presence of electrolytes the polysaccharide was brought down with 3 to 4 volumes of ethyl alcohol. When dissolved in 10 per cent trichloroacetic acid most of the material was obtained by the addition of 2 volumes of ethanol.

A 0.5 per cent solution of polysaccharide A Wood 46 in water yielded a pH of 6.40 when the pH of the water was 6.70. The acidic properties of polysaccharide A will be discussed in a forthcoming paper in connection with the electrophoresis.

Dialysis. Polysaccharide A passed through the cellophane membrane. The rate, however, was very slow, and the loss of material by dialysis for 2 days was almost negligible.

Gel filtration. The behaviour of polysaccharide A on Sephadex G 25 has been reported in (15, 16). Gel filtration with Sephadex G 50 Medium was carried out by the same technique. Egg albumin and ammonium sulphate served as references for colloids and salts respectively. Polysaccharide A was found to pass straight through the column with the albumin, but some polysaccharide A was found to overlap into the salt containing fractions. These fractions were refiltrated and this time the remaining polysaccharide A separated from the salts.

Ultraviolet absorption spectrum. The ultraviolet absorption spectrum of a purified sample of polysaccharide A Wood 46 has been presented in the preceding paper (16). The polysaccharide A Wood 46 preparation used for the present studies showed an almost identical spectrum without any peaks. The extinction of a 0.5 per cent aqueous solution was only 0.061 at 260 mμ and 0.051 at 280 mμ. The UV absorption

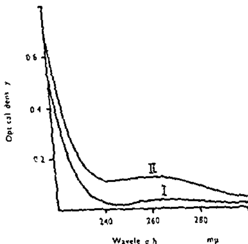


Fig. 1

Ultraviolet absorption spectra of polysaccharide A Wood 46 in 0.1N hydrochloric acid (I) and in 0.01N sodium hydroxide (II)

spectrum of this material was also recorded with 0.1N hydrochloric acid and 0.01N sodium hydroxide as solvents. A low peak now appeared at 260 mμ and 263 mμ in acidic and alkaline milieu respectively, with the corresponding minima at 246 mμ and 243 mμ (Fig. 1).

Relative viscosity. Polysaccharide A Wood 46, 0.5 per cent aqueous solution showed a relative viscosity of 1.13. Examined in 0.1M sodium chloride solution, the relative viscosity decreased to 1.06. The values have not been corrected for the densities (cf. Methods).

Specific optical rotation. Tubes of 20 cm length and a 0.5 per cent solution of polysaccharide A in water were used. The specific rotation of polysaccharide A Wood 46 at 21° C was —11.5°.

Biuret and *Dische*'s tests were carried out on 0.5 per cent solutions of polysaccharide A Wood 46 and were both negative. The *Molisch* test was very weakly positive on the same concentration of the polysaccharide and negative on a 0.1 per cent solution. A 0.1 per cent solution of polysaccharide A showed a negative *Bial*'s test, while a 0.5 *per cent* solution gave a positive reaction of about the same strength as given by 4 μg arabinose per ml.

Phosphorus. The content of organic phosphorus was 5.61 per cent. No inorganic phosphorus was found.

Nitrogen. The nitrogen content was 3.78 *per cent*. *To* make sure that all of the ammonium ions from the chromatography on Dowex-1 had been removed by gel filtration, two samples of polysaccharide A W[illegible] 46 [illegible]

I[illegible]

use [illegible] of reducing sugars, calculated as glucose, was 31.67 per cent, and calculated as *glucosamine* (free base) 31.30 per cent

mμ One hundred μg of glucosamine gave an extinction of about 0 55 with a 1 0 cm cell and a final volume of 10 ml The extinction values given by 20 to 100 μg of glucosamine usually showed a linear relationship to the weight of sugar, the value for 100 μg sometimes being a little below the straight line For quantities of glucosamine over 100 μg Beer's law was not strictly applicable The wavelength of maximum absorption shifted from 530 mμ to 510 mμ in the course of 5 days the drift being faster with greater amounts of glucosamine

The stability of the colour was examined with five duplicate standards of glucosamine and a hydrolyzed sample of polysaccharide A Wood 46 The colour given by 25 μg, remained almost unchanged for 24 hours at 530 mμ, and showed some increase at 505 mμ (Table I) The extinctions of the other standards decreased during the observation time, more pronouncedly with the higher values The hydrolyzed sample behaved like the glucosamine standards As a routine therefore the spectrophotometric recording is taken immediately after finishing the procedures and a second reading after 24 hours The latter reading has been performed as muramic acid shows a maximum at 505 mμ with considerably increased values after 24 hours (5) The quantities of material in the unknowns are regulated to give hexosamine values within 25 to 75 μg of glucosamine

RESULTS

General properties Polysaccharide A Wood 46 was a white, amorphous, rather hygroscopic powder It dissolved readily in water, yielding a clear colourless solution even in a concentration of 5 g per 100 ml

The material was not precipitated by trichloroacetic acid, mineral acids or alkali, and could not be salted out with ammonium sulphate or sodium chloride At pH 5 2 and in the presence of electrolytes the polysaccharide was brought down with 3 to 4 volumes of ethyl alcohol When dissolved in 10 per cent trichloroacetic acid most of the material was obtained by the addition of 2 volumes of ethanol

A 0 5 per cent solution of polysaccharide A Wood 46 in water yielded a pH of 6 40 when the pH of the water was 6 70 The acidic properties of polysaccharide A will be discussed in a forthcoming paper in connection with the electrophoresis

Dialysis Polysaccharide A passed through the cellophane membrane The rate, however, was very slow, and the loss of material by dialysis for 2 days was almost negligible

Gel filtration The behaviour of polysaccharide A on Sephadex G-25 has been reported in (15, 16) Gel filtration with Sephadex G-50 Medium was carried out by the same technique Egg albumin and ammonium sulphate served as references for colloids and salts respectively Polysaccharide A was found to pass straight through the column with the albumin, but some polysaccharide A was found to overlap into the salt-containing fractions These fractions were re-filtrated, and this time the remaining polysaccharide A separated from the salts

Ultraviolet absorption spectrum The ultraviolet absorption spectrum of a purified sample of polysaccharide A Wood 46 has been presented in the preceding paper (16) The polysaccharide A Wood 46 preparation used for the present studies showed an almost identical spectrum, without any peaks The extinction of a 0 5 per cent aqueous solution was only 0 061 at 260 mμ and 0 051 at 280 mμ The U V absorption

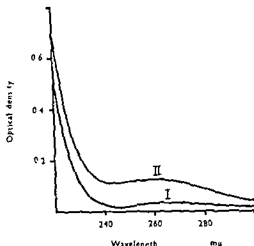


Fig 1
Ultraviolet absorption spectra of polysaccharide A Wood 46 in 0.1N hydrochloric acid (I), and in 0.01N sodium hydroxide (II)

spectrum of this material was also recorded with 0.1N hydrochloric acid and 0.01N sodium hydroxide as solvents A low peak now appeared at 260 mμ and 263 mμ in acidic and alkaline milieu respectively, with the corresponding minima at 246 mμ and 243 mμ (Fig 1)

Relative viscosity Polysaccharide A Wood 46, 0.5 per cent aqueous solution, showed a relative viscosity of 1.13 Examined in 0.1M sodium chloride solution, the relative viscosity decreased to 1.06 The values have not been corrected for the densities (cf Methods)

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Phosphorus The content of organic phosphorus was 5.61 per cent No inorganic phosphorus was found

Nitrogen The nitrogen content was 3.78 per cent To make sure that all of the ammonium ions from the chromatography on Dowex-1 had been removed by gel filtration, two samples of polysaccharide A Wood 46 were steam distilled with strong alkali No ammonia was released

Reducing sugars Both glucose and glucosamine hydrochloride

Hexosamines The hexosamine content was 23 9 per cent calculated as glucosamine, free base The hydrolyzed sample of polysaccharide A Wood 46 gave the same absorption spectrum, between 500 and 550 mμ, as glucosamine

Chemical data on several polysaccharide A preparations have been presented in Table 2 Some data given by *Wieghard & Julianelle* (36) have been included for comparison

TABLE 2

Analytical Data and Serological Activity of Different Polysaccharide A Preparations

Preparation number (see under Materials)	Relative viscosity 0 5 per cent in water*	[α]D degrees	Kjeldahl N per cent	Organic P per cent	Reducing sugars (as glucose) per cent	Hexosamines (as glucosamine free base) per cent	Molisch (0 1 per cent of material) + to ++++	Ring test titre
1	1 15	—28 3	7 88	4 29	20 6	10 3	+	1 1 × 10^6
2	1 12	—7 5	2 20	7 75	24 3	16 4	++	1 4 × 10^6
3			2 48	5 96	30 45	18 6	3 7% §	1 2 × 10^6
4	1 18	—10 0	5 82	5 57	34 41†	24 6	—	1 4 × 10^6
5	1 13	—11 5	3 78	5 61	34 67	23 9	—	1 4 × 10
W + J pol A″		+6 7	4 09	6 27	26 1		++++‖	1 6–8 × 10^6]

″ *Wieghard & Julianelle*'s polysaccharide A preparation (36)
* Determination in sodium chloride solution was carried out only with preparation number 5, figures obtained in watery solution are therefore presented
† One determination only due to shortage of material
§ Estimated by a quantitative method (24)
‖ Positive in high dilutions
] Carried out by a somewhat different precipitation method (23)

DISCUSSION

Polysaccharide A did not precipitate with agents ordinarily precipitating proteins Moreover, the negative biuret test and the low extinction value at 280 mμ indicate that the preparation is protein-free

Some of the physicochemical data suggest that the molecular weight of polysaccharide A is not very high The very low relative viscosity, the partial dialyzability through cellophane membranes, and the rapid migration in agar (15) The data obtained with Sephadex G 50 Medium, indicate a molecular weight above 8,000 to 10,000 (24) It has to be remembered, however, that the above-mentioned properties are not directly related to the molecular weight, but to the molecular size and form

Compared with the relative viscosities of pneumococcal polysaccharides (4), the values obtained with polysaccharide A are very low Figures that were too high were obtained when the determination was performed in aqueous solution This is probably due to Coulomb's forces, the electroviscous effect (24), which may be suppressed by the addition of salts

The UV absorption spectra in acidic and alkaline milieu showed absorption maxima corresponding to those of nucleic acids or purine-pyrimidine compounds, apparently caused by a contaminant Nucleic acids show very strong absorption at 260 mμ *Gale & Folkes* (12) calculated the amount of staphylococcal nucleic acids according to an extinction at 260 mμ of 280 for a 1 per cent solution in a 1 0 cm cell Only a trace of this contaminant, therefore, can be present The very weak reaction with Bial's test may be due to the same substance In either case the amount is very small, less than 0 1 per cent, and will not interfere with the data obtained from the chemical analyses

The hexosamine content, calculated as glucosamine, can account for only half the amount of nitrogen found Since the preparation is protein free, and almost free of nucleic acids, the most probable explanation is that the nitrogen difference is caused by peptides containing no aromatic amino acids, and thus producing no absorption at 280 mμ

Acid hydrolysis with 3N hydrochloric acid for 3 hours at 100° C was employed for the determination of both hexosamines and reducing sugars since the hexosamines seemed to constitute the major part of the reducing sugars, and qualitative tests for other sugars were negative It seems probable that the somewhat higher reducing values obtained on further hydrolysis were due to interfering substances (cf below)

While most nitrogen free sugars give a near-theoretical yield of free sugars on acid hydrolysis, the amino polysaccharides do not (33) Most naturally occurring amino ...
tyl residues ar...
results which ...
pound gives a negative or a weak Elson-Morgan reaction (11, 22) A corresponding product, ribitol glucosaminide, was found by *Armstrong et al* (1) on acid hydrolysis of *Staph aureus* teichoic acid Therefore the figures obtained for the hexosamine content are likely to be too low
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... by glucosamine was stable for 3 hours In our experiments (cf Methods) this held true only for the 25 μg standard *Boas* (3) advised against heating with acetylacetone at temperatures above 93° C, since incomplete formation of the acetylacetone condensation product might then ensue, and the colour spectrum might achieve an additional peak at 550 mμ By the procedure of *Rondle & Morgan* (30) condensation is carried out at 100° C for 20 minutes We have examined the absorption spectrum between 500 and 550 mμ with gluco-

samine standards and hydrolysates of polysaccharide A, but never observed an additional peak at 550 mμ

Glucosamine was found to be rather resistant to acid, in agreement with the findings of *Boas* (3)

The most troublesome cause of error in the Elson-Morgan reaction is the interfering colour given by mixtures of reducing sugars and amino acids (19) *This does not seem to have influenced our results,* since qualitative tests have revealed no sugars other than hexosamines

Two amino sugars have been found in the cell walls of *Staph aureus,* glucosamine and muramic acid (6, 25) The latter produces less colour than glucosamine at 530 mμ, but shows a maximum at 505 mμ with *increasing extinction values during the first 24 hours (5) The hydro*lysate of polysaccharide A Wood 46 gave the same absorption spectrum as glucosamine, and displayed no peak or increasing values at 505 mμ The amount of muramic acid, if present, must therefore be comparatively small

Glucosamine was found to have a greater reducing capacity than glucose, in accordance with the findings of *Neuberger* (27) The amount of reducing sugars calculated as glucosamine, was greater than the hexosamine content estimated by the Elson-Morgan reaction The other tests for carbohydrates, Molish, Dische, and Bial, however, showed no evidence of other sugars A similar discrepancy has been reported by *Salton & Pavlik* (32), who examined the carbohydrate content of cell walls of *Micrococcus lysodeikticus* The content of reducing sugars determined by the Hagedorn-Jensen method, was considerably higher than the content calculated from a summation of the amounts of the individual sugars They attribute the higher reducing values to a possible interaction with amino acids In our case, this must await further discussion until the preparation has been examined for amino acids Moreover, the identity of the hexosamine(s) *should be established, since* the various amino sugars produce different amounts of colour by the method of *Rondle & Morgan* (5, 30), and may show different reducing capacities (35)

If we presume that the hexosamine is N-acetylglucosamine and the phosphorus occurs as a phosphate, these two compounds will account only for less than 50 per cent of the molecule It seems therefore probable that polysaccharide A contains other components which have escaped detection by the methods employed

Comparing the chemical data on different polysaccharide A preparations, it appears that apart from the nitrogen content, the two preparations which have been purified on DEAE cellulose and Dowex-1, show almost identical chemical and physicochemical properties There are however, relatively great *variations between the other preparations* and the cause of this will be discussed after paper chromatographic examination of polysaccharide A As a result of purification the nitrogen values become lower and the phosphorus and hexosamine *values higher*

(cf preparations 1 and 2 in Table 2) The main difference between our polysaccharide A preparations and *Wieghard & Julianelle*'s (36) preparation is the strongly positive Molisch test obtained by these investigators Most probably the material which gives the Molisch reaction is a contaminant *Wieghard & Julianelle* (36) have not identified their sugar component(s), but they found that the greater part of the reducing sugars was non fermentable

SUMMARY

Chemical analyses of polysaccharide A have revealed high contents of hexosamines and organic phosphorus, 23 9 and 5 61 per cent, respectively No other sugars were detected by qualitative tests The polysaccharide showed a low laevorotation, a very low relative viscosity and acidic properties It was found to be protein free and almost free of nucleic acids The nitrogen content, however, was higher than might be accounted for by the hexosamine content, and only less than half the molecule could be attributed to a hexosamine-phosphate compound Polysaccharide A has therefore, to be studied further by paper chromatographic methods to reveal other possible components which might have escaped detection by the methods employed in the present study

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IMMUNOCHEMICAL STUDIES ON POLYSACCHARIDE A OF STAPHYLOCOCCUS AUREUS

4 *Paper Chromatography of Acid Hydrolysates*

By

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Received 9 ii 62

Chemical analysis of polysaccharide A have revealed high contents of organic phosphorus and of hexosamines (12) No other sugars were demonstrated by qualitative tests, and the polysaccharide was practically free of proteins and nucleic acid materials Apart from a negative or weakly positive Molisch test the chemical analyses showed great similarity to the data on the polysaccharide A material of *Wieghard & Julianelle* (25)

In the present work hydrolysates of polysaccharide A have been studied by paper chromatographic methods An acid hydrolysate of a *Staphylococcus aureus* wall teichoic acid has been included as a reference for ribitol and anhydroribitol (2)

MATERIALS

1 Crude polysaccharide A from st... [illegible]

[illegible]

... wex 1 columns ... preceding paper (12), the teichoic acid sample from *Staph aureus* H was kindly furnished by Professor *J Baddiley* Newcastle (3)

METHODS

General Procedures

The chromatogram were run by the descending method and Whatman filter paper no 1 was employed almost exclusively For qualitative measures the papers were used as supplied unwashed.

The material was introduced onto the paper as a circular spot of 7 mm diameter to improve the resolution of partly overlapping spots "windows" were cut between the application areas producing narrower bands After the material had been applied the papers were hung inside the chromatography chamber for at least two hours to obtain equilibrium with the atmosphere saturated with solvent vapours The solvent was applied to the trough through a hole without removing the glass cover When the solvent had run a concenient distance 32 to 35 cm in 12 to 24 hours depending on the solvent used the papers were removed from the chamber and the

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3 The ammoniacal silver nitrate reagent of *Partridge* (17) was used for reducing sugars

4 Addition of equal volumes of 2N sodium hydroxide to the ammoniacal silver nitrate above rendered the reagent very sensitive to sugar alcohols (8)

5 The sodium periodate benzidine reagents (6) also very sensitive to sugar alcohols were preferred in most instances to reagent 4 for the study of these substances

6 The hexosamine reagents of *Partridge* (18) *i e* the Elson Morgan reaction modified for paper chromatograms were used for amino sugars and their N acetyl derivatives A weak yellow or salmon red colour was sometimes observed on glycine spots

7 When treatment with acetylacetone was omitted from the procedures in reagen ...

as

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EXPERIMENTAL AND RESULTS

Amino Acids and Amino Sugars

The hydrolysates of polysaccharide A Wood 46 were first run in the two phenol solvent systems and sprayed with the ninhydrin reagent 1 and the hexosamine reagents 6 and 7 (Table 1 and 2)

TABLE 1

The R_F Values of Ninhydrin Reacting Spots of Three Acid Hydrolysates of Polysaccharide A Wood 46 (cf under Methods) and some Markers in Phenol Water (4 1)

	Spot number				
	1	2	3	4	5
Hydrolysate I	0 12	0 17	–	0 36	0 55
Hydrolysate II	0 14	0 19	–	0 38	0 58
Hydrolysate III	0 14	0 19	0 26	0 38	0.56
Glucosamine hydrochloride	–	0 18	–		–
Glutamic acid			0 27	–	
Glycine	–	–		0.36	–
Alanine	–			–	0 58

TABLE 2

The R_F Values of Spots of Acid Hydrolysates of Polysaccharide A Wood 46 (cf under Methods) Treated with the Hexosamine Reagent (a) and Spots Treated with the Same Reagent when Condensation with Acetylacetone was Omitted (b)

	Spot number			
	1	2	3	4
Hydrolysate I a	0 11	0 16	0 49	*0 70*
Hydrolysate I b	*0 10*		*0.51*	0 71
Hydrolysate II a	0 11	0 17	0 54	–
N Acetylglucosamine		–	–	*0 71*

Solvent system Phenol water (4 1) Figures in italics denote blue spots indicating N acetyl amino sugars The other spots were red

solvent boundary marked with a pencil The sheets were then dried for 5 to 10 minutes in an oven at 100° C On two dimensional separation after developing in the first solvent system, drying was carried out in an air stream at 30 to 40° C.

The papers were sometimes examined under an ultraviolet lamp (2015 Chromatolite 2537 A Shandon) for fluorescent or absorbing areas before being sprayed with a colour reagent The spots were outlined in pencil and the nature and intensity of the colour noted immediately, since most colours were apt to fade with time The room temperature varied from 16 to 22° C which influenced the R_F values When unknown spots were identified by tracer substances the travelling distances were always compared directly on the same chromatogram

Acid Hydrolysis

Three hydrolysates were prepared from polysaccharide A Wood 46

Hydrolysate I Polysaccharide A was heated for 20 minutes in 2N sulphuric acid in a boiling water bath as recommended by (20), for the demonstration of N acetyl amino sugars

Hydrolysate II To study the sugars and sugar alcohols hydrolysis was carried out with 3N hydrochloric acid at 100° C for 3 hours in closed tubes

Hydrolysate III For the identification of amino acids the material was hydrolyzed with 6N hydrochloric acid at 105° C for 16 hours in sealed ampoules

The sulphuric acid of hydrolysate I was neutralized to pH 4 5 with barium hydroxide and the precipitated barium sulphate centrifuged off Hydrolysates II and III containing hydrochloric acid were evaporated to a sirup by gentle heating at low pressure The residues were re dissolved in water and re evaporated twice and taken to dryness in a desiccator containing silica gel concentrated sulphuric acid and sodium hydroxide pellets The dried materials were made up to a concentration of 2 per cent in water

The teichoic acid sample was treated as hydrolysate II

Developing Solvents

A Phenol water (4 1, w/v) (7) A colourless phenol reagent was obtained by distilling Phenolum (Merck) with shreds of aluminium foil at atmospheric pressure

B Phenol water ammonia (7) The phenol water mixture (A) was applied to the trough while 3 per cent aqueous ammonia was placed at the bottom of the jar

To prevent discoloration of the solvent fronts due to oxidation of phenol 0 01 per cent of the di sodium salt of ethylenediaminetetraacetic acid (Titriplex *III* Merck) was sometimes added to the phenol reagent

Two dimensional separation was achieved with the solvent systems employed by *Salton & Pavlil* (21)

C Pyridine water (4 1 v/v) and

D n butanol acetic acid water (6 1 2 v/v)

For the detection of ribitol and anhydroribitol (2)

F n Propanol ammonia (d 0 88) water (6 3 1 v/v) and

I n butanol ethanol water ammonia (d 0 88) organic phase (40 10 49 1 v/v)

The organic layer was applied to the trough while the aqueous layer was run onto the bottom of the jar

For the identification of glycine by the o phthalaldehyde reagent the chromatogram was run according to *Patton & Foreman* (19) in

G Ethanol 77 per cent by volume

Spray Reagents

1 Ninhydrin 0 1 per cent in water saturated n butanol (7) was used for amino sugars and amino acids

2 Ninhydrin 0 4 per cent in n butanol containing 10 per cent of phenol (5) This reagent was more sensitive than the ninhydrin reagent above and enabled the detection of 0 1 to 0 2 µg of glycine

Both ninhydrin reagents were acidified by acetic acid

mined by paper chromatography in various solvent systems and amino acid tracers with about the same mobility were run parallel herewith (Table 4)

TABLE 4

The R_F Values of Four Amino Acid Spots Obtained from Hydrolysate III (cf under Methods) of Polysaccharide A Wood 46 The Spots Had Been Eluted from a Chromatogram Run in Phenol Water (4 1)

	Solvent system (cf Methods)					
	A	B	C	E	F	G
Spot 1	0.26	0.22		0.18	0.05	
Glutamic acid	0.27	0.22	–	0.19	0.05	–
Cystine	–	0.36		0.21	–	–
Spot 2	0.40	0.47	0.20	0.32	0.10	0.33*
Glycine	0.39	0.47	0.20	0.32	0.11	0.33*
Serine		0.41		–	–	–
Spot 3	–	0.85		0.28	–	–
Lysine	–	0.85		0.29	–	–
Ornithine	–	0.79	–	–	–	–
Spot 4			0.34	0.42	0.17	–
Alanine	–		0.35	0.41	0.16	–

* The chromatogram was sprayed with the o phthalaldehyde reagent (10) and the two spots showed a green colour and brownish fluorescence

All other spots were obtained with the ninhydrin reagent

Spot 1 was identified as glutamic acid In accordance with the behaviour of acidic amino acids its travelling rate was slowed by the addition of ammonia to the phenol water system It separated well from cystine in solvent system B

Spot 2 was found to be glycine showing travelling distances identical to the glycine tracer in several systems though positioned relatively close to the serine tracer The identity was confirmed by the production of the typical green spot and brown fluorescence when run in system G and treated with the o phthalaldehyde reagent

Spot 3 was identified as lysine Its mobility increased considerably on the addition of ammonia to system A indicating a basic amino acid Satisfactory separation from ornithine was obtained

Spot 4 which reacted both with the ninhydrin and the hexosamine reagents was found to contain alanine as the major ninhydrin reacting component Alanine was well separated from the spot suggesting muramic acid in system C in accordance with the findings of *Strominger & Threnn* (24)

Two dimensional chromatography was carried out in the solvent systems C and D The paper was sprayed with the n [illegible]

[illegible] papers were treated with the

Two spots reacted with ninhydrin only, and their travelling rates suggested the presence of glutamic acid and glycine The spot with R_F of about 0.53 reacted with both the ninhydrin and hexosamine reagents and is suggestive of muramic acid (22). Alanine showed a similar mobility, and may be present together with the amino sugar

Spot 2 (in both tables) was by far the largest and reacted strongly with both the ninhydrin and hexosamine reagents. Its travelling rate agreed with that of the glucosamine tracer.

Spot 4 in Table 2 reacted strongly with both hexosamine reagents but not with ninhydrin *Its blue colour and position indicated a N-acetyl* hexosamine, obviously the N-acetyl derivative of the major amino sugar of spot 2.

The slow-*moving spot 1 did not correspond to any amino sugar* known to us It might possibly be an oligosaccharide or another incomplete hydrolysis product (11, 20). This spot and the spot suggestive of muramic acid (cf. above) also reacted with reagent 7 for N-acetyl hexosamines

Additional procedures are needed for a more rigorous identification of amino sugars, and will be reported in the next article.

TABLE 3

The R_F Values of Ninhydrin-Reacting Spots of Acid Hydrolysates of Polysaccharide A Wood 46 (cf. under Methods) and some Markers in Phenol-Water Ammonia (Solvent System B)

	Spot number			
	1	2	3	4
Hydrolysate I	–	0.44	0.59	–
Hydrolysate III	0.22	0.45	0.60	0.80
Glutamic acid	0.22	–	–	–
Glycine	–	0.44		
Glucosamine	–	–	0.58	–
Lysine		–		0.82

The R_F values of spots of a chromatogram run in the phenol-water-ammonia system have been presented in Table 3 The major amino sugar now migrated to an R_F of about 0.60, *being liberated* as the free base No other spots were detected in the position which was occupied by this amino sugar in system A, but a new spot appeared with the R_F of lysine

In order to identify the amino acids, the spots were eluted and examined in various solvent systems Also two-dimensional chromatography was carried out

Hydrolysate III was applied along a 40 cm *straight line* and the chromatogram run in solvent system A Strips were cut parallel to the application line corresponding to the amino *acid* spots, guided by a ninhydrin-treated strip, and eluted with water The eluates were exa-

pared to glycerol and the increase in anhydroribitol and decrease in ribitol on further hydrolysis are all in agreement with the findings of the above authors

Spot 3 is most probably the same compound as *Salton & Pavlik* (21) found after hydrolysis of cell walls with 6N hydrochloric acid for 16 hours at 105° C showing an $R_{anhydroribitol}$ of 1 5 in a n butanol acetic acid water system A compound showing the same chromatographic behaviour was obtained when ribitol or anhydroribitol was treated in the same way They conclude that this substance most likely is a dianhydroribitol

Some weakly reacting spots near the starting point were found in hydrolysates of polysaccharide A and teichoic acid apparently representing other ribitol derivatives (2) The hydrolysates were examined also in solvent system A where the same three new components were found No other sugars were detected

Examinations of other Polysaccharide A Preparations

Some paper chromatographic studies have been performed on other polysaccharide A preparations Since however these preparations were relatively impure the results are of limited value

A crude polysaccharide A preparation from strain 1503 was found to give strong amino acid spots suggestive of threonine leucine and isoleucine in addition to the 5 amino acids of polysaccharide A Wood 46

A polysaccharide A preparation from strain 1503 which had been purified by chromatography on DEAE cellulose columns (cf Materials) on the other hand gave few and very weak amino acid spots

DISCUSSION

Paper chromatographs of acid hydrolysates of polysaccharide A Wood 46 has revealed the same amino acids as found by several investigators in the cell wall mucopeptide of *Staph aureus* (9 15 23) The amino acids of polysaccharide A will be estimated quantitatively in order to compare their molar ratios with those of the cell wall amino acids

Three amino sugar spots have been detected two of which seem to be glucosamine (or galactosamine) and muramic acid Additional steps to identify the amino sugars will be performed The amino sugars glucosamine and muramic acid in addition to the amino acids mentioned above constitute the components of the mucopeptide of *Staph aureus* cell walls (9 23) Therefore it seems probable that polysaccharide A Wood 46 contains a mucopeptide moiety

The [illegible]
the hig [illegible]
serolog [illegible] ed from *Staph aureus* and
polysaccharide A (13) seem to indicate that polysaccharide A contains

ninhydrin reagent 2 The spot travelled a distance which was a little shorter than the distance travelled by glycine, but generally it was covered by the comparatively greater glycine spot

Hydrolysate III and the eluates containing amino acids were run in solvent system E and sprayed with Folin's reagent according to *Mü* . (16) Proline and hydroxyproline give red spots with this reagent N adiitonal spots, however, were detected

The hydrolysate of *Staph aureus* H teichoic acid gave two disti spots corresponding to glucosamine and alanine when treated wit ninhydrin, and in addition a weak, unidentified spot which when compared with the findings of *Armstrong et al* (2) might be ribitol glucosaminide The alanine content seemed to be much greater than in polysaccharide A, while the glucosamine spot agreed in strength with the major amino sugar spot of polysaccharide A

Other Sugars and Sugar Alcohols

The hydrolysates of polysaccharide A and teichoic acid were compared chromatographically in several solvent systems The papers were treated with the silver nitrate reagents and the sodium periodate reagents In addition to the amino sugar spots two new, distinct spots were detected in the hydrolysates of polysaccharide A Identically positione spots were found in the teichoic acid hydrolysate A third, very fast moving spot appeared in hydrolysate III, and this spot was not found in the other hydrolysates The R_F values of these new spots in two solvent systems have been presented in Table 5

TABLE 5

The R_F Values of Spots Detected by the Periodate Benzidine Reagents which Had Not Reacted with the Ninhydrin and Hexosamine Reagents

	Solvent system I			Solvent system I		
	Spot number			Spot number		
	1	2	3	1	2	3
Hydrolysate I	0 45	(0 58)		0 24	(0 36)	
Hydrolysate II	0 46	0 56		0 23	0 36	–
Hydrolysate III	(0 46)	0 57	0 75	(0 25)	0 37	0 73
Hydrolysate of teichoic acid	0 46	0 57		0 22	0 36	
Glycerol	–				0 40	

Figures in brackets indicate very weak spots

As compared with the findings of *Armstrong et al* (1, 2), spots 1 and 2 undoubtedly correspond to ribitol and anhydroribitol respectively Our R_F values were somewhat lower than values reported by the authors, who, however, used the ascending *method* The relative positions of the spots, the slightly lower mobility of anhydroribitol as com-

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Part II Observations on the acidic hydrolysis of derivatives of 2 amino-

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20

21

22 *Strange R E & Kent L H* The isolation characterization and chemical syn-

23 Stron ... ion of the cell wall ... hanism of action of

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25 H ... logical specifity of staphylo- ... the chemical nature of the soluble specific substances J Exper Med., 62 23 30 1935

a teichoic acid moiety Visually estimated the amount of ribitol seemed to be the same in polysaccharide A and teichoic acid hydrolysates This, in addition to the high content of organic phosphorus, shows that the teichoic acid moiety constitutes the greater part of the polysaccharide

The teichoic acid of *Staph aureus* cell walls has been found to be a polymer of ribitol phosphate with N-acetylglucosamine and D-alanine residues (2, 4) In our preparation the amount of alanine appeared to be considerably smaller than the amount in the teichoic acid sample It has to be established whether the alanine of polysaccharide A is readily split off by alkali as in teichoic acid or forms a part of the mucopeptide moiety It is also of great interest to find whether the teichoic acid and the mucopeptide moieties of polysaccharide A are linked together These investigations will be reported in a later article

The presence of amino acids in acid hydrolysates of polysaccharide A Wood 46 may explain the nitrogen content which could not be accounted for by the hexosamine content (12) More accurate figures will be obtained after quantitative estimation of the amino acids and the individual amino sugars

The crude polysaccharide seemed to contain more amino acid material than the purified preparations This may explain the high nitrogen content and the positive biuret test shown by this preparation The polysaccharide A 1503 preparation which had been purified on DEAE cellulose, contained apparently very small amounts of amino acids, which is in accordance with the low nitrogen content of this preparation (12) Apparently the amount of the mucopeptide moiety varies for some reason

SUMMARY

Five amino acids, glutamic acid, glycine, lysine, alanine, and serine, three amino sugars, and the sugar alcohol ribitol have been demonstrated by paper chromatography of acid hydrolysates of polysaccharide A Wood 46 The polysaccharide is supposed to be composed of two structural components, a mucopeptide and a ribitol teichoic acid, both resembling the corresponding structures of the staphylococcal cell walls The teichoic acid undoubtedly constitutes the greater part of the polysaccharide

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Solvent systems A phenol water (4 1 w/v) B phenol water (A) with 1 per cent ammonia added at the bottom of the chamber C phenol saturated with a 0 067M phosphate buffer of pH 12 (15) D n butanol ethanol water ammonia (d 0 88) (4 4 1 1 v v) E pyrid ne water (4 1 v/v) F n propanol ammonia (d 0 88) water (6 3 1 v/v)

Spray reagents 1 0 1 per cent ninhydr n in water saturated n butanol 2 0 4 per cent ninhydr n *in n butanol w th 10 per cent phenol added (3) 3 the hexosamine* reagents of *Partr dge* (18) 4 anil ne hydrogen phthalate (19) 5 ammoniacal silver nitrate (17)

Oxilat on of the am no sugars w th ninhydrin was carried out by the method descril ed by *Gardell et al* (7)

Glucosamine was N acetylated by the technique described by *Roseman & Ludowieg* (23)

2 ur pe ce it acet c ac d was added to the ninhydrin reagent for spraying the papers developed in alkaline solvents After spraying the papers were all wed to dry

EXPERIMENTAL AND RESULTS

Identification and Quantitative Determination of Amino Sugars

Amino sugars of polysaccharide A 1503 In one experiment the hydrolysate of a crude polysaccharide material from stra n 1503

our and 2 2 ml fractions were collect d The peak volume of the major hexosamine component was 83 ml in one experiment and 81 ml in another The peak volume of the glucosamine reference was 83 ml and that of galactosamine 96 ml This strongly indicated that the major hexosamine component was glucosamine The identity of this hexosamine was also established by paper

FROM THE UNIVERSITY OF BERGEN, SCHOOL OF MEDICINE
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IMMUNOCHEMICAL STUDIES ON POLYSACCHARIDE A OF STAPHYLOCOCCUS AUREUS

5 Identification of Amino Sugars Quantitative Determination of Amino Sugars and Amino Acids

By

GUNNAR HAUKENES

Received 22 ii 62

Paper chromatographic studies on hydrolysates of polysaccharide A indicated that the major structural component of the polysaccharide was a ribitol teichoic acid (11) In addition five amino acids were found These were the same as those present in acid hydrolysates of staphylococcal cell walls (13, 25)

This paper deals with the identification of the amino sugars of polysaccharide A, and the quantitative determination of amino sugars and amino acids

MATERIALS

1 Crude polysaccharide A 1503 prepared as described in (12)
2 Polysaccharide A 1503 purified on DEAE cellulose as described in (8)
3 Polysaccharide A Wood 46 purified on DEAE cellulose and Dowex 1 columns (9) the same batch as was used for chemical analyses and paper chromatographic studies

METHODS

Acid hydrolysis The materials were hydrolyzed with 3N hydrochloric acid for 3 hours at 100° C for the examination of sugars and with 6N hydrochloric acid for 16 hours at 105° C for the study of amino acids The technique has been described in more detail in the preceding paper (11)

Ion exchange chromatography The strong cation exchange resins Amberlite IR 120 and Zeo Karb 225 were used for the separation of amino sugars (*Gardell* 1953) The resins were prepared in the hydrogen form and eluted with 0 33N hydrochloric acid as described by *Crumpton* (4) A 1 0 × 30 cm column of Amberlite IR 120 100 230 mesh or a 1 2 × 45 cm column of Zeo Karb 225 > 200 mesh both 8 per cent cross linked was used

The fractions were examined for hexosamines and in some experiments by the Molish test for other sugars as well The effluent volumes at which the amino sugars were eluted including the fraction containing the greatest amount of the sugar the so called peak volumes were recorded The peak volume of an amino sugar relative to that of glucosamine the $R_{glucosamine}$ value was calculated as it is a characteristic feature of an amino sugar (4)

Hexosamines were estimated by *Rondle* & *Morgan*s method (22)

Paper chromatography Details of the technique have been given in the previous paper (11)

Solvent systems A phenol water (4 1, w/v), B phenol water (A) with 1 per cent ammonia added at the bottom of the chamber, C, phenol saturated with a 0 067M phosphate buffer of pH 12 (15), D, n butanol ethanol water-ammonia (d 0 88) (4 4 1 1 v/v), F pyridine water (4 1, v/v), F, n propanol ammonia (d 0 88)-water (6 3 1, v/v)

Spray reagents 1, 0 1 per cent ninhydrin in water saturated n butanol, 2, 0 4 per cent ninhydrin in n butanol with 10 per cent phenol added (3), 3, the hexosamine reagents of *Partridge* (18), 4, aniline hydrogen phthalate (19), 5 ammoniacal silver nitrate (17)

Oxidation of the amino sugars with ninhydrin was carried out by the method described by *Gardell et al* (7)

Glucosamine was N-acetylated by the technique described by *Roseman & Ludowieg* (23)

Four per cent acetic acid was added to the ninhydrin reagent for spraying the papers developed in alkaline solvents ... papers were allowed to dry

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EXPERIMENTAL AND RESULTS

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Amino sugars of polysaccharide A 1503 In one experiment the hydrolysate of a crude polysaccharide material from strain 1503 ...

... per hour, and 2 2 ml fractions were collected The peak volume of the major hexosamine component was 83 ml in one experiment, and 81 ml in another The peak volume of the glucosamine reference was 83 ml and that of galactosamine 96 ml This strongly indicated that the major hexosamine component was glucosamine The identity of this hexosamine was also established by paper

chromatography of the oxidation products after treatment with ninhydrin The hexosamine was denatured with ninhydrin (7) and subsequently examined in the two phenol systems A and B, parallel with galactosamine and glucosamine references, treated in the same way, and with arabinose After oxidation three spots appeared with the aniline hydrogen phthalate reagent According to *Stoffyn & Jeanloz* (26) these spots represented the unchanged amino sugar, the pentose resulting from the ninhydrin-degradation, and a fast-moving intermediate compound The oxidation product of the major amino sugar of polysaccharide A showed a migration rate identical to the one of arabinose (Table 1), which confirms that the unknown amino sugar is glucosamine and not galactosamine

TABLE 1

The R_F Values of the Oxidation Products of Glucosamine Galactosamine and the Major Hexosamine Component of Polysaccharide A 1503 Compared with Arabinose

Solvent system	R_F values of spots obtained from			
	Arabinose	Glucosamine ninhydrin degraded	Hexosamine from polysaccharide A ninhydrin degraded	Galactosamine ninhydrin degraded
		0.18	*0.18*	*0.08*
				0.19
A	0.51	0.51	0.52	0.46
		0.67	0.69	0.67
	0.54	0.53	0.54	0.51
B		*0.59*	*0.61*	?
		0.77	0.77	0.79

Figures in italics denote spots reacting with the hexosamine reagents of *Partridge* (18) All spots reacted with the aniline hydrogen phthalate reagent

In another experiment several groups of fractions were isolated by the Elson Morgan reaction and the Molisch test (Table 2) The polysaccharide A material used for this experiment had been purified twice on DEAE cellulose columns

Fractions 5 7, representing material not retained by the resin showed a relatively strong Molisch reaction On the basis of our present knowledge of the composition of polysaccharide A, two groups of compounds might be expected to pass straight through the column, N-acetyl amino sugars if present, and sugar alcohols However, neither of these substances react in the Molisch test In a previous paper *(10)* the Molisch reaction of several polysaccharide A preparations *has been* reported Highly purified preparations were obtained showing a negative Molisch test, while some preparations with the same serological

reactivity showed a positive reaction This sugar can accordingly be removed without impairing the serological reactivity The Molisch-reacting material obtained in this experiment represents most probably a contaminant, and its identity was not established

TABLE 2

The $R_{glucosamine}$ Values of Sugars of a Hydrolyzed Sample of Polysaccharide A 1503 Separated on Amberlite IR 120 and their Reaction in the Molisch Test and the Hexosamine Test of Rondle & Morgan (22)

Fraction number	Peak volume ml of 0 33 N HCl	$R_{glucosamine}$ value	Molisch test	Hexosamine test (Rondle & Morgan)
5 to 7	13	0 16	+	—
11 to 15	28	0 34	—	+
16 to 18	37	0 45	+	—
30 to 45	81	0 98	—	+
Glucosamine	83	1		
Galactosamine	96	1 16		

The hexosamine-reacting fractions 11–15 appeared in the eluate with an $R_{glucosamine}$ value of 0 34, a value which does not correspond to any of the amino sugar values listed by *Crumpton* (4) On paper chromatography a slow-moving spot was demonstrated in the phenol systems, agreeing with the unknown amino sugar spot described previously (11) The colour spectrum in the reaction of *Rondle & Morgan* (22) showed a maximum at 525 mμ when read after 30 minutes, and a maximum at 510 mμ after 24 hours (Fig 1) The spectrum showed some resemblance to both the glucosamine and muramic acid spectra (cf Fig 3) [illegible]
acid for 2 a [illegible]

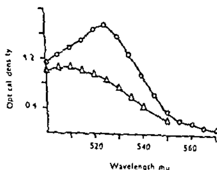


Fig 1

Absorption spectrum of the unhydrolysed residue of polysaccharide A 1503 in the *Rondle & Morgan* hexosamine reaction (22)

○—○ Readings made after 30 minutes
△—△ Readings made after 24 hours

paper chromatographically in phenol systems A and B and the papers were treated with the ninhydrin reagent 1 the hexosamine reagents and the ammoniacal silver nitrate reagent Both of the hydrolysates yielded the same spots *indicating glucosamine glutamic acid muramic* acid and some unhydrolyzed material Two dimensional chromatography was performed with phenol water (A) in one direction and n butanol ethanol water ammonia (D) at right angles Owing to the slow migration rate in system D the solvent was allowed to run off the paper which had been serrated at the lower edge The chromatogram was sprayed with ninhydrin and the identity of the above mentioned components was confirmed In addition some very weak ninhydrin reacting spots were found probably representing traces of other amino acids

The third group fractions 16 18 which gave a very weak Molisch reaction showed in Rglucosamine value of 0 45 The substance was not identified because of shortage of material

Fractions 30 45 which contained glucosamine gave an additional spot with the R_f of muramic acid on paper chromatography Its peak volume was not accurately recorded but seemed to be a little greater than that of glucosamine The two substances were separated by paper chromatography in phenol water (A) The evaporated and concentrated material from fractions 30 45 was treated along a straight line on a paper and developed in the phenol water system The two widely separated components were eluted from the paper with water The identity of *muramic acid* was *confirmed* by its typical behaviour in the *Rondle & Morgan*'s reaction (4 22) It showed considerably higher extinction values at 505 mμ than at 530 mμ The extinction at 505 mμ increased greatly during 24 hours while it remained constant at 530 mμ The amount of muramic acid was calculated on the basis of the observation by *Crumpton* (4) that at 530 mμ muramic acid produced only 27 1 per cent of the colour of an equal weight of glucosamine when estimated by *Rondle & Morgan*'s method (22) Only the relative amounts of the amino sugars of this polysaccharide A preparation were estimated The molar ratio of glucosamine to muramic acid was 2 9 to 1 0 Calculated as glucosamine the quantity of material in fractions 11 15 amounted to about one *third of the glucosamine content*

Amino sugars of polysaccharide A Wood 46 The amino sugars of a hydrolysate of 10 mg polysaccharide A Wood 46 were separated on a 1 2 × 15 cm column of Zeo Karb 225 by the technique described above The flow rate was regulated to 4 ml per hour and 2 5 3 0 ml fractions were collected Three hexosamine reacting components were obtained (Fig 2) The column was also run with galactosamine and glucosamine references to calculate the Rgl values of the components of the hydrolysate and of galactosamine The components were examined also by paper chromatography (Table 3) and their absorption spectra in the *Rondle & Morgan* reaction (22) were recorded (Fig 3) A sample

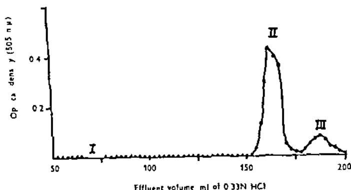


Fig 2

Separation of the hexosamine components of a hydrolysate of polysaccharide A Wood 46 on Zeo Karb 225 The extinction values at 505 mμ in the *Rondle & Morgan* reaction (22) read after 24 hours have been depicted since the values for muramic acid at 530 mμ were very low The curves therefore are not representative of the amounts of the amino sugars Fractions from 0 to 50 ml and from 200 to 280 ml gave a negative reaction for hexosamines and have not been included

I Unidentified component (cf the text) The fractions produced a very weak colour which was not recorded spectrophotometrically until the fractions had been collected and concentrated
II Glucosamine
III Muramic acid

of components II was acetylated and showed the same R_F value in system A as N acetylglucosamine

It is apparent from Table 3 that the three components correspond to the three hexosamine reacting spots obtained on the paper chromatograms which were described in the preceding paper (11) Component I showed an $R_{glucosamine}$ value corresponding to fractions 16–18 of polysaccharide A 1503 and gave a spectrum which showed greater resemblance to that of muramic acid than that of glucosamine The substance was resistant to further hydrolysis with 6N hydrochloric acid and its identity was not established Its colour spectrum in the Elson Morgan reaction however showed some resemblance to the spectrum of fractions 11 15 of polysaccharide A 1503 and the substance may represent an unhydrolyzed residue or for example ribitol glucosaminide which was found by *Armstrong et al* (1) in acid hydrolysates of *Staph aureus* teichoic acid The colour spectrum of the latter substance however was not reported

Component II is glucosamine being represented by the strongly reacting spot on paper chromatograms treated with ninhydrin and the hexosamine reagents of *Partridge* (18)

Although no authentic sample of muramic acid was available for comparison there is no doubt that component III was muramic acid Its $R_{glucosamine}$ value and colour spectrum agreed with the findings of *Crumpton* (4) and the paper chromatographic mobility in phenol water (A) was the same as that obtained by *Strange & Kent* (27)

paper chromatographically in phenol systems A and B, and the papers were treated with the ninhydrin reagent 1, the hexosamine reagents and the ammoniacal silver nitrate reagent Both of the hydrolysates yielded the same spots, *indicating glucosamine, glutamic acid*, muramic acid and some unhydrolyzed material Two-dimensional chromatography was performed with phenol-water (A) in one direction and n-butanol-ethanol-water-ammonia (D) at right angles Owing to the slow migration rate in system D the solvent was allowed to run off the paper, which had been serrated at the lower edge The chromatogram was sprayed with ninhydrin, and the identity of the above-mentioned components was confirmed In addition some very weak ninhydrin reacting spots were found, probably representing traces of other amino acids

The third group, fractions 16–18, which gave a very weak Molisch reaction, showed an $R_{glucosamine}$ value of 0 45 The substance was not identified because of shortage of material

Fractions 30 45, which contained glucosamine, gave an additional spot with the R_F of muramic acid on paper chromatography Its peak volume was not accurately recorded, but seemed to be a little greater *than that of glucosamine The two substances were separated by paper* chromatography in phenol-water (A) The evaporated and concentrated material from fractions 30–45 was treaked along a straight line on a paper and developed in the phenol-water system The two widely separated components were eluted from the paper with water The identity of muramic acid was confirmed by its typical behaviour in the *Rondle & Morgan*'s reaction (4, 22) It showed considerably higher extinction values at 505 mμ than at 530 mμ *The extinction at 505 mμ* increased greatly during 24 hours, while it remained constant at 530 mμ The amount of muramic acid was calculated on the basis of the observation by *Crumpton* (4) that, at 530 mμ, muramic acid produced only 27 1 per cent of the colour of an equal weight of glucosamine, when estimated by *Rondle & Morgan*'s method (22) Only the relative amounts of the amino sugars of this polysaccharide A preparation were estimated *The molar ratio of glucosamine to muramic acid* was 2 9 to 1 0 Calculated as glucosamine the quantity of material in fractions 11–15 amounted to about one third of the glucosamine content

Amino sugars of polysaccharide A Wood 46 The amino sugars of a hydrolysate of 10 mg polysaccharide A Wood 46 were separated on a 1 2 × 45 cm column of Zeo-Karb 225 by the technique *described above* The flow rate was regulated to 4 ml per hour, and 2 5 3 0 ml fractions were collected *Three hexosamine-reacting components* were obtained (Fig 2) The column was also run with galactosamine, and glucosamine references to [illegible] values of the components of the hydrolysat[illegible] were examined also by paper [illegible] sorption spectra in the *Rondle & Morgan* reaction (22) were *recorded* (Fig 3) A sample

Amino Acid Content

Glutamic acid Preliminary experiments indicated a content of 0 5 to 1 per cent in the hydrolysate of polysaccharide A Wood 46, and standards corresponding to these amounts were used Glutamic acid gave a small, well defined spot in the phenol-water systems, and the content was estimated to be 0 8 per cent

Glycine Visually estimated the content of glycine was found to range between 2 and 3 per cent Subsequently the content was estimated twice in system E and once in system C with the following results 2 6, 2 7 and 2 6 per cent

Alanine According to preliminary experiments standards corresponding to 2 and 3 per cent were selected The determination was performed in system E, and a concentration of alanine of 2 2 per cent was found

Lysine Several estimations were performed in the phenol water systems B and C, where lysine separated well from the other amino acids with an R_F of about 0 80 Some degree of discoloration, however, was always recorded in this region, and it was difficult to get a representative filter paper blank for the adjustment of the base line The lysine content in these experiments was estimated visually to be a little below 1 7 per cent In system F the content was estimated as 1 7 per cent

The molar ratios, glycine alanine lysine (glutamic acid) were 3 0 2 1 1 0 (0 5), using glycine as unity

The amount of serine was too small to be determined quantitatively by this method The optical configuration of the amino acids was not investigated

DISCUSSION

Two amino sugars, glucosamine and muramic acid, have been identified in acid hydrolysates of polysaccharide A The distinction of glucosamine from galactosamine has been discussed under Experimental As pointed out by *Salton & Pavlik* (25) mannosamine cannot be distinguished from glucosamine by paper chromatographic methods, including oxidation with ninhydrin Mannosamine will be eluted in the fractions containing muramic acid from a Zeo-Karb 225 column (4) The colour spectrum of mannosamine in the *Rondle & Morgan* reaction (22) however, is identical to that of glucosamine and accordingly different from that of muramic acid The colour spectrum of the f[illegible]

[illegible] chromatography of these fractions

Polysaccharide A 1503 contained relatively large amounts of an incompletely hydrolyzed material composed of [illegible]

acid and glutamic acid The colour [illegible]

[illegible]son Morgan reaction showed great [illegible]

ride described by *Perkins* (20) This [illegible] was liberated from

TABLE 3

The $R_{glucosamine}$ Values on Elution from Zeo-Karb 225, the R_F Values in Solvent System A and the Absorption Maxima in the Rondle & Morgan reaction (22) of three Hexosamine Components of Polysaccharide A Wood 46

Hexosamine component see figure 2	Peak volume ml of 0 33N HCl	$R_{glucosamine}$ value	R_F value solvent system A	Absorption max after hours	
				0 mμ	24 mμ
I	71	0 42	0 10	520	505
II	167	0 99	0 18	530	525
III	*191*	*1 13*	0 53	505	505
Glucosamine	169	1	0 18	530	525
Galactosamine	202	1 20			

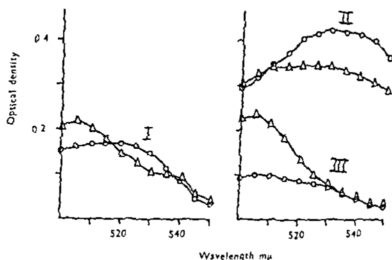


Fig 3

Absorption spectra in the *Rondle & Morgan* hexosamine reaction (22) of three hexosamine components of a hydrolysate of polysaccharide A Wood 46 (cf Fig 2)

I Unidentified substance (cf the text) II Glucosamine III Muramic acid
○——○ Readings made immediately — — Readings made after 24 hours

The three groups of fractions were evaporated to dryness and their hexosamine contents estimated The hydrolysate which was applied to the column, was thus found to contain 19 8 per cent of glucosamine, and 7 8 per cent of muramic acid, the latter being calculated as above The amount of component I was 1 2 per cent, calculated as glucosamine, and 4 4 per cent calculated as muramic acid

The hexosamine content of this polysaccharide A preparation, calculated as glucosamine, was 23 9 per cent when estimated before separation of the components (10) Thus almost quantitative recovery was obtained after separation

The molar ratio glucosamine muramic acid was 3 6 1 0 in polysaccharide A Wood 46

If we presume that the teichoic acid moiety of polysaccharide A is a N-acetylglucosamine ribitol phosphate compound, this will account for 76 per cent of polysaccharide A Wood 46. The calculation is based upon the phosphorus content of polysaccharide A and that of the *Staph aureus* teichoic acid investigated by *Baddiley et al* (2). The main objection to this calculation is, that, although too low values apparently were obtained for the glucosamine content, it is uncertain whether equimolecular amounts of glucosamine and ribitol phosphate are present in polysaccharide A. The total amount of muramic acid (calculated as N-acetylmuramic acid) and amino acids was 16.4 per cent. The actual amount of the hexosamine-reacting residue has not been estimated. Corrections for water uptake during hydrolysis, however, will give slightly lower values for the mucopeptide content. With the above reservations about 90 per cent of polysaccharide A Wood 46 has been accounted for.

The nitrogen contents of the amino acids and the amino sugars of polysaccharide A Wood 46 amounted to 3.24 per cent. The Kjeldahl-nitrogen of the same material was estimated to be 3.78 per cent in previous experiments (10). Thus about 90 *per cent of the nitrogen has* been recovered.

SUMMARY

Polysaccharide A Wood 46 was found to contain 19.8 per cent of glucosamine and 7.8 per cent of muramic *acid*. The *total amount* of amino acids was 7.3 per cent. The molar ratios of the *amino acids* and muramic acid differed to a certain degree from those *reported for* staphylococcal cell walls.

The analytical data obtained suggested a recovery of about 90 *per* cent of the polysaccharide and most of the nitrogen content could be *accounted for*.

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walls of *Micrococcus lysodeikticus* and *Staph aureus* by lysozyme and was found to be composed of N-acetylglucosamine and N-acetylmuramic acid The presence of the above-mentioned hexosamine-reacting compound in hydrolysates of polysaccharide A indicates that the hydrolysis has not been complete The figures for the content of hexosamines will be too low, since unsplit compounds and hydrolysis products give less colour in the hexosamine reaction than the free sugars (5, 22) The amount of residue seemed to be considerably higher in the hydrolysate of polysaccharide A 1503 than in polysaccharide A Wood 46 This may account for the varying amounts of sugars in different polysaccharide A preparations (cf Table 2 in (10)) More accurate figures might be obtainable with stronger hydrolysis Some destruction of the hexosamines will ensue, and the amount of ammonia formed has to be estimated

The mucopeptide moiety constituted a very small part of polysaccharide A In order to obtain distinct amino acid spots for quantitative determination, 100 to 200 µg of the hydrolysate had to be applied to the paper Owing to this the glucosamine spot became large and frequently showed trailing, which complicated the recording of the colour density Moreover, overlapping ribitol compounds seemed to influence the shape of the amino acid spots in some solvent systems The accuracy of the method used for quantitative determination of amino acids depended therefore, primarily on the selection of spots of the same width for scanning It was also found to be essential to make a preliminary visual estimation of the approximate amount of each amino acid, in order to select standards differing little in amount from the unknowns

Somewhat different molar ratios have been reported for the amino acids of the cell walls of *Staph aureus* (13, 14, 25) As a rule, equimolecular amounts of glutamic acid and lysine were found, and 3–5 moles of glycine and 2 3 moles of alanine per mole of lysine The mucopeptide of polysaccharide A showed the molar composition glycine alanine lysine (glutamic acid) of 3 0 2 7 1 0 (0 5), thus it contained comparatively small amounts of glutamic acid The muramic acid content of the cell walls has regularly been found to be equimolar to glutamic acid and lysine The molar value of the muramic acid of polysaccharide A was 2 7 relative to lysine It should be remembered, however, as pointed out by Salton (24), that the molar ratios of the cell walls represent only the gross composition The mucopeptide of polysaccharide A, on the other hand, is most likely a fragment of the walls As to our muramic acid value, reservation has to be made since the method was not controlled with an authentic sample of the muramic acid

Baddiley et al (1961) found that the molar ratio alanine phosphorus was 0 66 1 0 in the teichoic acid preparation obtained by extracting walls of *Staph aureus* H with cold trichloroacetic acid In polysaccharide A Wood 46 the corresponding ratio was 0 14 1 0 It will be examined and reported in a later paper whether the alanine linkage in polysaccharide A, like that of the teichoic acids, is labile in *alkaline* solution

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DISSIMILATION OF C^{14} LABELLED GLUCOSE BY NEISSERIA MENINGITIDIS

I The Formation of CO and Acetate from Glucose Carbon

By

K. JYSSUM

Received 5 1 62

In previous experiments it has been demonstrated that cells from Neisseria meningitidis contain the enzyme systems necessary for a dissimilation of glucose through the conventional Embden Meyerhof route (*Jyssum, Borchgrevink & Jyssum* 1961) Meningococci are also equipped with enzymes which perform the substrate transfers of an oxidative pentose pathway as well as those of the Entner Doudoroff cleavage (*Jyssum & Jyssum* to be published) Studies of the over all oxidation of glucose to CO_2 and acetic acid indicate that *glucose is oxid ized further down than to the acetate level* The "secondary" oxidation of glucose however, does not proceed completely to CO_2 and water (*Jy* [illegible]
T [illegible]
thr [illegible]
ally [illegible]
med [illegible] the first communication the incorporation into the major end products of glucose catabolism has been measured

MATERIALS AND METHODS

The methodology and experimental manipulations employed in the [illegible] tions were analogous to those [illegible] micrebe was the meningococc [illegible]
$C^{14}O_2$ respirometry Oxidat [illegible] in Warburg vessels Fluted fi [illegible] from which $C^{14}O_2$ was to be [illegible] free KOH was placed into the [illegible] 20 per cent [illegible] [illegible] was washed with ethanol water [illegible] to aluminium planchets and assayed for radioactivity with a Frieseke and Hoepfner windowless gasflow counter Determinations were made

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TABLE 1

Distribution of Radioactivity from C 1 and C 6 of Glucose among some Metabolic Products and Cellular Fractions of Neisseria meningitidis

Material analysed	Per cent of activity of glucose after 120 minutes incubation	
	Glucose 1 C^{14}	Glucose 6 C^{14}
CO	[illegible]4.5	3.6
Steam volatile material	<0.1	26.3
Ether soluble fraction	0.3	1.8
TCA precipitable material	0.7	14.6

[illegible] fraction was determined after wet combustion

These results indicate that while the C–1 is rapidly decarboxylated C 6 is more slowly metabolized to CO and acetate the major end products of glucose catabolism in Neisseria meningitidis (*Jyssum Borchgrevink & Jyssum* 1961) A further analysis of the labelling of various substances found in the ether soluble and in the TCA precipitable fractions is reported in a following communication (*Jyssum* 1962)

Determinations of data on specific activities of substances isolated from variously labelled glucoses allow the estimation of catabolism pathways provided corrections are made for the endogenous metabolism (*Lewis Blumenthal Weinrach & Weinhouse* 1955) Experiments were accordingly started to investigate the relative specific activities (RSA) of CO and acetate generated by suspensions of N meningitidis metabolizing labelled glucose

These investigations were limited to experiments with short incubation times This was done in order to decrease the influence of "secondary pathway" reactions In the presence of 0 15 M inorganic phosphate the primary oxidation of glucose has been found to take place at a very rapid rate while the secondary pathway functions only slowly (*Jyssum Borchgrevink & Jyssum* 1961 *Jyssum & Joner* 1962) Under the conditions chosen for the present assay it is accordingly assumed that the secondary pathway contributes to the formation of CO and acetate to a very small probably insignificant extent

A series of experiments was arranged such as recorded in Table 2 The experiments with glucose U C^{14} were run in order to obtain material for a correction for the production of CO and acetate from endogenous material In these analyses it is assumed that the specifically labelled glucose carbons are diluted to a similar extent hence permitting a calculation of a corrected relative specific activity (*Lewis Blumenthal Weinrach & Weinhouse* 1955)

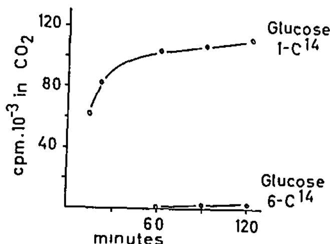


Fig 1.

The release of CO_2 from carbon 1 and carbon 6 of glucose in suspensions of N meningitidis actively catabolizing labelled glucose
The Warburg vessels contained in 3 2 ml 300 μmoles Tris as buffer pH 7 4 1 ml meningococcus suspension, 0 2 ml 10 per cent KOH in the center well and 4 μmoles labelled glucose which was tipped in from the sidearm at zero time
The gas phase was air, and the temperature 37°

to a standard deviation of 5 per cent, and corrected for background Corrections for self absorption were performed by a graphical method when necessary (*Colowick & Kaplan* 1957) Coincidence loss was too small to require any correction under the conditions of the assay

Incorporation into acetate After deproteinization with sulfuric acid the volatile acids were removed by steam distillation in a Markham still Acetic acid was recovered by neutralization of the acid in the steam distillate with $Ba(OH)_2$ The solution was evaporated to dryness and counted In some experiments duplicates were run in which the activity in the steam distillate was determined as $BaCO_3$ after wet combustion with a persulfate method (*Chen & Lauer* 1957) No significant difference was found between the two methods

Chemicals C^{14} labelled compounds were obtained from The Radiochemical Centre Amersham Bucks, G B

RESULTS

Whole cells were permitted to respire on glucose 1-C^{14} and glucose 6-C^{14} for various periods of time in order to obtain orientation concerning any preferential oxidation of either of these carbons of glucose In Figure 1 the activities in the CO_2 recovered have been recorded These data clearly demonstrate that the susceptibility of the first carbon of glucose to oxidative attack is strikingly greater than that of the sixth carbon atom

Experiments were next performed to explore the fate of the sixth carbon Some results have been recorded in Table 1 It is seen that a considerable amount of radioactivity is recovered in the steam volatile fraction, while a much smaller amount was found in CO_2 Pronounced activity was also found in the ether soluble fraction The protein fraction had also become significantly labelled during the same period of incubation

TABLE 1

Distribution of Radioactivity from C-1 and C-6 of Glucose among some Metabolic Products and Cellular Fractions of Neisseria meningitidis

Material analysed	Per cent of activity in glucose after 120 minutes incubation	
	Glucose 1 C^{14}	Glucose 6 C^{14}
CO_2	74 5	3 6
Steam volatile material	<0 1	26 3
Ether soluble fraction	0 3	1 8
TCA precipitable material	0 7	1 46

Th... H 7.2 150 µmoles Tris ... ml 0 2 ml 10 per cent ... was tipped in from the ... temperature 37° The reaction was stopped with sulfuric acid when steam distillation was to be performed otherwise with TCA The activity of the TCA precipitable material as well as that of the ether soluble fraction was determined after wet combustion

These results indicate that while the C–1 is rapidly decarboxylated C 6 is more slowly metabolized to CO_2 and acetate the major end pro- ... *Borch* ... various ... ble frac... reported in a following communication (*Jyssum* 1962)

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These investigations were limited to experiments with short incubation times This was done in order to decrease the influence of secondary pathway reactions In the presence of 0 15 M inorganic phosphate the primary oxidation of glucose has been found to take place at a very rapid rate while the secondary pathway functions only slowly (*Jyssum Borchgrevink & Jyssum* 1961 *Jyssum & Joner* 1962) Under the conditions chosen for the present assay it is accordingly assumed that the secondary pathway contributes to the formation of CO_2 and acetate to a very small probably insignificant extent

A series of experiments was arranged such as ... The experiment ... for a corre... material ... glucose ... ...at extent hence permitting a calculation of a corrected relative specific activity (*Lewis Blumenthal Weinrach & Weinhouse* 1955)

TABLE 2

Incorporation of C-1 and C-6 from Glucose into CO_2 and Acetate in Experiments with N. meningitidis

Substrate			Product analysed after 30 minutes					
			CO_2			Acetate		
Compound	μmoles	Activity cpm	μmoles	RSA	Corrected RSA	μmoles	RSA	Corrected RSA
Glucose U-C^{14}	4	1.70×10^5	6.6	65.2		3.36	72.6	
Glucose 1-C^{14}	4	1.56×10^5	6.3	89.8	138	3.28	0.09	0.3
Glucose 6-C^{14}	4	1.13×10^5	6.5	1.8	2.8	3.32	39.4	120

Each Warburg vessel contained 300 μmoles P_i as buffer pH 7.2; otherwise the system was as described in the legend of Table 1. The CO_2 evolved was determined by the Warburg indirect technique with corrections for the absorption in the buffer. The contents from five identical systems were combined and analysed for acetate. The analytical procedures and calculations were as described under methods.

A comparison of CO_2 production from glucose U-C^{14}, glucose 1-C^{14} and glucose 6-C^{14} may give some indication as to the pathway operation in glucose catabolism, even if such data are not conclusive (*Agranoff, Brady & Colodzin* 1954).

The high RSA of the respiratory CO_2, obtained during the metabolization of glucose 1-C^{14} by meningococci is characteristic of the pentose or Entner-Doudoroff paths. The data on incorporation of glucose 6-C^{14} into CO_2 are consistent with this idea. If glucose carbon six was exclusively converted to the methyl carbon of pyruvate, its conversion to CO_2 might be expected to be slower than that of the other carbons. The RSA of 2.8 per cent in the present experiments may be the result of such a localization of the radioactivity.

The incorporation of labelled radioactivity in acetate corroborates this hypothesis. Any acetate derived from glucose 1-C^{14} by the Embden-Meyerhof process should have a corrected RSA of 150 per cent, while the Entner-Doudoroff process or the pentose path would not yield labelled acetate from glucose 1-C^{14}. In the former the carbon of C-1 is lost on decarboxylation of pyruvate, and in the latter in pentose phosphate formation. The observed corrected RSA of 0.3 per cent may be taken to indicate that no more than a very small part of the acetate ($0.3 \times 100/150 = 0.2$ per cent) produced from glucose came via the Embden-Meyerhof process.

Acetate arising from glucose 6-C^{14} via the pentose path should have a very high specific activity, probably of at least 150 per cent (*Lewis, Blumenthal, Weinrach & Weinhouse* 1955). Acetate arising via the Entner-Doudoroff process should also have an activity of 150 per cent. The observed value of 120 per cent thus may indicate the operation of either one of these mechanisms, or perhaps rather a simultaneous operation of both.

DISCUSSION

The assumption that CO_2 and acetate are the predominant and quantitatively the only significant end products of glucose catabolism in resting cells of Neisseria meningitidis is corroborated by the present tracer experiments.

The glucose-1 carbon, though incorporated readily into CO_2, is incorporated into acetate at a low rate, and contributes only to a limited extent to the formation of various compounds which may be extracted from the cells in an ether soluble fraction. This carbon of glucose also provides little of the radioactivity incorporated into macro-molecules by resting cells. In contrast to this stands the failure of the glucose-6 carbon in labelling the respiratory CO_2, and its heavy labelling of acetate as well as the other fractions mentioned.

In the arrangement of these experiments, and in the interpretation of the data obtained advantage is taken of the fact that meningococci perform the reactions of the "primary" glucose catabolism rapidly in the presence of 0.15 M inorganic phosphate, while the "secondary" oxidation is strongly inhibited by such an addition (*Jyssum, Borchgrevink & Jyssum* 1961, *Jyssum & Joner* 1962). Little information as to the pathway mechanisms could be gained if, in the absence of inhibitors, the substrate were oxidized to completion. In short time incubations with meningococci, and in the presence of inorganic phosphate, however, the radioactive CO_2 and acetate produced are thought to originate mainly from reactions of the primary pathways.

The mechanism by which the "secondary" oxidative pathway of glucose oxidation in meningococci is thought to be blocked by high concentrations of inorganic phosphate is discussed in forthcoming publications (*Jyssum* 1962, *Jyssum & Joner* 1962).

The release of CO_2 from specifically labelled glucose demonstrates a preferential oxidation of the first carbon of glucose. Investigations of relative specific activities (RSA) in CO_2 and acetate are taken to indicate that the major part of glucose is metabolized by way of the pentose phosphate pathway, by the Entner Doudoroff cleavage, or by a simultaneous operation of both. The data indicate that less than 0.2 per cent of substrate glucose is broken down via the Embden-Meyerhof route of glycolysis.

SUMMARY

CO_2 and acetate produced from labelled glucose by suspensions of Neisseria meningitidis have been examined. Relative specific activities (RSA) have been calculated and corrected for endogenous metabolism.

The production of CO_2 from carbon 1 was always higher than that from the average carbon atom of glucose, while CO_2 production from carbon 6 was much lower.

Acetate from glucose-1-C^{14} had a very low corrected RSA while that from glucose-6-C^{14} had a high corrected RSA.

The results show that the Embden-Meyerhof process occurs to a very small extent. The data are indicative of an operational pentose phosphate oxidation, an Entner-Doudoroff cleavage, or of a simultaneous function of both.

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DISSIMILATION OF C^{14} LABELLED GLUCOSE BY NEISSERIA MENINGITIDIS

2. *The Incorporation of 1-C^{14} and 6-C^{14} into Cellular Components in Short-Time Experiments*

By

K. Jyssum

Received 5.1.62

The specific labelling of CO_2 and acetate, metabolic end products in *suspensions of* Neisseria meningitidis respiring on glucose-1-C^{14} and glucose-6-C^{14}, shows that the Embden-Meyerhof process occurs to a *very small extent*. The data are also indicative of an operational pentose *phosphate oxidation*, an Entner-Doudoroff cleavage, or of the simultaneous function of both (*Jyssum* 1962).

In the present experiments the actual incorporation *from the same two carbons of glucose* into various *intermediates* of the "primary" and of the "secondary" pathways *of glucose catabolism* have been observed after several short periods of exposure to glucose with very high specific activity.

MATERIALS AND METHODS

The same basic meth[illegible]

[illegible] (…activity required) and ... [co]ntents were mixed by a rapid rotary *movement*. With an automatic pipette ... ml samples were pipetted into pyrex tubes each containing 3 ml of absolute ethanol standing in a water bath at 80°. The time of removal of samples was measured with a stop watch. The hot ethanolic suspensions ... minutes ... [coo]led and ... [illegible]
aqueous ethanol [illegible]
[illegible]
[o]bservation of [illegible]

[illegible] supernatant
[illegible] 1960). The
[illegible] a gentle stream of N_2. When

the ether phase had been evaporated the acids were neutralized before the final evaporation to dryness The material was transferred to paper and analysed by chromatography

Incorporated amino acids The precipitates from the extractions with hot aqueous ethanol were suspended in 1 ml 6 N HCl in flame sealed tubes, and the tubes left at 110° for 20 hours After cooling the tubes were opened and the contents gently evaporated to dryness The material was extracted with ethanol water and aliquots of the extracts were used for further analysis by chromatography

Chromatographic procedures The samples were applied to the origin position of sheets of Whatman No 4 chromatographic paper (35 cm × 35 cm) The papers were analysed by descending chromatography in phenol formic acid (Kornberg 1958) The papers were air-dried and rechromatographed in the second direction in butanol propionic acid-water (Calvin & Benson 1949) In both directions the solvent was allowed to flow to within ½ cm from the edge

Radioautography and assay The air dried chromatograms were marked with radioactive indian ink and the location of the radioactive materials was determined by exposing the chromatogram to 35 cm × 35 cm Blue Brand X ray film for a suitable period (Usually 14 days) The labelled areas were outlined in pencil on the chromatogram and the radioactivity was measured by direct radioassay on the paper with the thin window gas flow counter of Frieseke and Hoepfner standing on a mask of thin lead sheet into which a square 2 cm × 2 cm had been cut Each area was counted for at least 3000 counts total, and the counts corrected for background radioactivity

Identification of labelled compounds The labelled compounds on the chromatogram were approximately identified in the two solvents used Furthermore they were eluted from the paper with water and co-chromatographed in appropriate solvent systems with authentic unlabelled samples of the suspected compounds These chromatographic procedures were generally the same which previously have been described (Jyssum Borchgrevink & Jyssum 1961, Jyssum & Jyssum 1962) Analyses of eluates from the spots corresponding to various phosphorylated compounds were also performed after treatment with phosphatase Dephosphorylation was carried out with Polidase S (Schwartz Labs Inc N Y) according to the procedures of Benson Bassham & Calvin (1951) The resulting compounds were chromatographed two-dimensionally

RESULTS

Incorporation into phosphorylated compounds

Analysis of the area on the chromatograms known to contain the sugar phosphates showed that a considerable number of substances had been labelled, some in very short periods To obtain more reliable data concerning the relative distribution of activity only spots with more heavy labelling and with good separation were further analysed Spots corresponding to sugar diphosphates, sugar monophosphates, 6 phosphogluconate, and phosphoenolpyruvate were sufficiently well separated to permit a comparison by direct counting on the paper The activity in these spots was compared with activity of the whole ethanol soluble material, minus activity of the residual glucose spot

Several data obtained by this technique are recorded in Table 1 From these data it becomes evident that a considerable amount of the activity incorporated from the glucose carbons 1 and 6 into the ethanol soluble fraction during the first minute is residing in various phosphorylated compounds

These experiments do not permit of comparisons to be made of the actual activities in these compounds at various times They do allow,

however, of conclusions to be drawn as regards the relative activities in different spots

In the case of both carbons tested the relative amount of activity in the phosphorylated compounds is decreasing after the first few seconds

These data consist with the conventional hypothesis concerning the role played by phosphorylated compounds in glucose catabolism

TABLE 1

Short Time Incorporation of C 1 and C-6 from Specifically Labelled Glucose into Various Phosphorylated Compounds in Neisseria meningitidis

Substrate	Substance analysed	Incubation period (seconds) Percentage distribution					
		5	10	15	20	30	60
Glucose 1 C14	Sugar diphosphates	0.2	0 5	0 5	0 6	1 1	0 7
	Sugar monophosphates	61 3	44 7	37 2	34.3	28 2	24 6
	6 phosphogluconate	22 8	34.3	43 1	41 8	41.2	38 2
	Phosphoenolpyruvate	–	–	–	–	–	–
	Total in these spots	84.3	79 5	80 8	76 7	70 5	63 5
Glucose 6 C14	Sugar diphosphates	3 5	2 8	2 5	1 8	1 5	0 5
	Sugar monophosphates	50 3	2[illegible] 4	11 5	9 9	8 5	7 0
	6 phosphogluconate	18 6	27.3	21 9	15 1	10 9	9 1
	Phosphoenolpyruvate	0 5	4 6	9 2	8 5	7 7	6 5
	Total in these spots	72 9	57 1	45 1	35 3	28 6	23 1

Solutions of specifically labelled glucose were added at [illegible]

Another important feature of the present experiments is the pronounced difference found in the labelling pattern from the two carbons of glucose under study

In the experiments with 1 C14 the phosphorylated compounds tested contain a significantly higher percentage of the activity in the total ethanol soluble fraction than is the case with 6-C14 Also, the C-1 does not label the PEP spot in the way it is done by C-6 Beside the PEP spot several non identified spots, tentatively classified as phosphorylated compounds were labelled from C-6 but not from C-1 [illegible] mulation in sugar [illegible] pears to precede [illegible] experiments it bee[illegible] incorporated from [illegible] other compounds of the ethanol soluble fraction than those actually measured The sugar monophosphates and 6 phosphogluconate spots appear to be intermediates in the metabolism of both carbons, while PEP may be an intermediate of C-6 but hardly of C-1 in the pathway of glycolysis

the ether phase had been evaporated the acids were neutralized before the final evaporation to dryness The material was transferred to paper and analysed by chromatography

Incorporated amino acids The precipitates from the extractions with hot aqueous ethanol were suspended in 1 ml 6 N HCl in flame-sealed tubes and the tubes left at 110° for 20 hours After cooling the tubes were opened and the contents gently evaporated to dryness The material was extracted with ethanol water and aliquots of the extracts were used for further analysis by chromatography

Chromatographic procedures The samples were applied to the origin position of sheets of Whatman No 4 chromatographic paper (35 cm × 35 cm) The papers were analysed by descending chromatography in phenol-formic acid (Kornberg 1958) The papers were air-dried and rechromatographed in the second direction in butanol-propionic acid water (*Calvin & Benson* 1949) In both directions the solvent was allowed to flow to within ½ cm from the edge

Radioautography and assay The air dried chromatograms were marked with radioactive indian ink and the location of the radioactive materials was determined by exposing the chromatogram to 35 cm × 35 cm Blue Brand X ray film for a suitable period (Usually 14 days) The labelled areas were outlined in pencil on the chromatogram and the radioactivity was measured by direct radioassay on the paper with the thin window gas-flow counter of Frieseke and Hoepfner standing on a mask of thin lead sheet into which a square 2 cm × 2 cm had been cut Each area was counted for at least 1000 counts total and the counts corrected for background radioactivity

Identification of labelled compounds The labelled compounds on the chromatogram were approximately identified in the two solvents used Furthermore they were eluted from the paper with water and co chromatographed in appropriate solvent systems with authentic, unlabelled samples of the suspected compounds These chromatographic procedures were generally the same which previously have been described (*Jyssum Borchgrevink & Jyssum* 1961, *Jyssum & Jyssum* 1962 Analyses of eluates from the spots corresponding to various phosphorylated compounds were also performed after treatment with phosphatase Dephosphorylation was carried out with Polidase S (Schwartz Labs Inc N Y) according to the procedures of *Benson Bassham & Calvin* (1951) The resulting compounds were chromatographed two dimensionally

RESULTS

Incorporation into phosphorylated compounds

Analysis of the area on the chromatograms known to contain the sugar phosphates showed that a considerable number of substances had been labelled, some in very short periods To obtain more reliable data concerning the relative distribution of activity only spots with more heavy labelling and with good separation were further analysed Spots corresponding to sugar diphosphates, sugar monophosphates 6 phosphogluconate, and phosphoenolpyruvate were sufficiently well separated to permit a comparison by direct counting on the paper The activity in these spots was compared with activity of the whole ethanol soluble material, minus activity of the residual glucose spot

Several data obtained by this technique are recorded in Table I From these data it becomes evident that a considerable amount of the activity incorporated from the glucose carbons 1 and 6 into the ethanol soluble fraction during the first minute is residing in various phosphorylated compounds

These experiments do not permit of comparisons to be made of the actual activities in these compounds at various times They do allow,

however of conclusions to be drawn as regards the relative activities in different spots

In the case of both carbons tested the relative amount of activity in the phosphorylated compounds is decreasing after the first few seconds

These data consist with the conventional hypothesis concerning the role played by phosphorylated compounds in glucose catabolism

TABLE 1

Short Time Incorporation of C 1 and C-6 from Specifically Labelled Glucose into Various Phosphorylated Compounds in Neisseria meningitidis

Substrate	Substance analysed	Incubation period seconds Percentage distribution					
		5	10	15	20	30	60
Glucose 1 C^{14}	Sugar diphosphates	0 2	0 5	0 5	0 6	1 1	0 7
	Sugar monophosphates	61.3	44 7	37 2	34.2	28 2	24 6
	6 phosphogluconate	27 8	34 3	43 1	41 8	41.2	38 2
	Phosphoenolpyruvate	–		–	–	–	–
	Total in these spots	84 3	79 3	80 8	76 7	70 5	63 5
Glucose 6 C^{14}	Sugar diphosphates	3 5	2 8	2 5	1 8	1 5	0 5
	Sugar monophosphates	50.3	22 4	11 5	9 9	8 5	7 0
	6 phosphogluconate	18 6	27 3	21 9	15 1	10 9	9 1
	Phosphoenolpyruvate	0 5	4 6	9 2	8 5	7 7	6 5
	Total in these spots	72 9	57 1	45 1	35.3	28 6	23 1

Solutions of specifically labelled glucose were added at [illegible]
s spen [illegible]

Another important feature of the present experiments is the pronounced difference found in the labeling pattern from the two carbons of glucose under study

In the experiments with 1 C^{14} the phosphorylated compounds tested contain a significantly higher percentage of the activity in the total ethanol soluble fraction than is the case with 6 C^{14} Also the C-1 does not label the PEP spot in the way it is done by C-6 Beside the PEP spot several non identified spots tentatively classified as phosphorylated compounds were labelled from C-6 but not from C-1 The accumulation in sugar monophosphates from C-1 as well as from C-6 appears to precede the accumulation in [illegible]
experiments it becomes apparent th[illegible]
incorporated from C 6 than from [illegible]
ethanol soluble fraction than those [illegible] measured The sugar monophosphates and 6-phosphogluconate spots appear to be intermediates in the metabolism of both carbons while PEP may be an intermediate of C-6 but hardly of C-1 in the pathway of glycolysis

TABLE 2

Short-Time Incorporation of C-1 and C-6 from Specifically Labelled Glucose into some Organic Acids in Neisseria meningitidis

Substrate	Conditions of assay	Organic acid analysed	Incubation period (seconds) Percentage distribution					
			5	10	15	20	30	60
Glucose 1-C14	Tris buffer 0.15 M	Pyruvic	99.6	98.1	96.7	95.8	94.5	92.3
		Lactic	0.2	1.4	2.7	3.9	3.7	4.3
		Total in these spots	99.8	99.5	99.4	99.7	98.2	96.6
Glucose 1-C14	Phosphate buffer 0.15 M	Pyruvic	98.4	92.8	91.1	85.1	78.5	58.2
		Lactic	1.2	2.6	2.8	2.6	2.3	2.4
		Citr Isocitr			–	–	–	
		Succin Fumar					1.4	11.2
		Malic		–		1.2	3.6	9.8
		Total in these spots	99.6	95.4	93.9	88.9	85.8	81.6
Glucose 6-C14	Tris buffer 0.15 M	Pyruvic	44.6	39.1	33.8	28.5	20.1	18.9
		Lactic	0.2	0.6	0.5	1.8	1.4	1.8
		Citr Isocitr	52.4	51.8	51.2	45.8	41.8	21.2
		Succin Fumar			4.3	7.1	10.6	17.7
		Malic		–		2.2	4.3	14.6
		Total in these spots	97.2	93.5	89.8	85.4	78.2	74.2

The experimental system was the same as that described in the legend of Table 1. In the phosphate experiments the tris buffer was replaced by a phosphate buffer of the same molarity.

Incorporation into organic acids

The comparatively large amount of the radioactivity of the total ethanol soluble fraction which is residing in non-phosphorylated compounds requires an analysis of the distribution in other metabolic intermediates. Among the compounds found in the aqueous ethanol extract the organic acids play an important part. In the second series of experiments these acids were accordingly isolated, and their radioactivity compared. One feature of the experimental system used is that OAA is decarboxylated to pyruvic acid. The pyruvic acid, on the other hand appears to be stable provided the ether extract is not evaporated completely to dryness until it has been carefully neutralized. Volatile acids are removed by the procedure. The activity distribution among spots corresponding to the tricarboxylic and dicarboxylic acids of the TCA cycle, and to pyruvic and lactic acids were determined and compared with the total activity of the ether soluble fraction.

Some activity distributions obtained in short-time incubations with meningococcal cells have been recorded in Table 2. When considering

the data of the experiments without added P_i a remarkable difference is again found between the picture given by C-1 and that of C-6 from glucose Practically all the activity of the first carbon is found in two organic acids, namely in pyruvic acid and lactic acid, and in such a way that more than 90 per cent is found in the former Activity from the sixth carbon is on the other hand distributed to a number of organic acids which are known as intermediates of the TCA cycle

A consideration of the succession in which the tricarboxylic and dicarboxylic acids are labelled from glucose 6-C^{14} may offer some information regarding the way the TCA cycle operates in meningococci Within the first few seconds after the addition of the labelled material considerable activity from the sixth carbon has already accumulated in the tricarboxylic acids and in pyruvate In contrast to this, the dicarboxylic acids start to become labelled after a significant lag In the experiments recorded this lag lasted for approximately 20 seconds During the second half a minute, however, considerable radioactivity accumulated in these acids

In Table 2 an experiment has been included also which illustrates the effect of added exogenous P_i on the labelling of the TCA intermediates It is seen that glucose 1-C^{14} in the presence of 0 15 M P_i confers some radioactivity to the dicarboxylic acids during the second half a minute of incubation It does not, however, label the tricarboxylic acids This shows that in cells which actively catabolize glucose, the secondary ' oxidation by way of the TCA cycle intermediates is significantly changed by the addition of P_i

Incorporation into amino acids of macromolecules

The material which was precipitated with hot aqueous ethanol was analysed mainly to explore the incubation time necessary for an incorporation of glucose carbon into macromolecules With the present knowledge of the biosynthetic pathways it was also considered likely that the way in which the amino acids were first labell [illegible] some informat [illegible]

From [illegible]
acids in [illegible] as early as 5 seconds after the addition of labelled glucose During the first minute of these incorporation experiments, however, the number was small of substances which were labelled Most of the activity of the hydrolysate was found in 7 spots Among these 5 were identified as amino acids as recorded in the table The other two spots which were not identified, were labelled from glucose 1 C^{14} as well as from glucose 6-C^{14}

It is seen that alanine and valine were labelled from C-1 of glucose during the first 20 seconds of incubation These two amino acids on the whole account for the major part of the activity incorporated into the hydrolysate from this carbon during the first minute of incubation

TABLE 2

Short Time Incorporation of C-1 and C-6 from Specifically Labelled Glucose into some Organic Acids in Neisseria meningitidis

Substrate	Conditions of assay	Organic acid analysed	Incubation period (seconds) Percentage distribution					
			5	10	15	20	30	60
Glucose 1-C^{14}	Tris buffer 0.15 M	Pyruvic	99.6	98.1	96.7	95.8	94.5	92.3
		Lactic	0.2	1.4	2.7	3.9	3.7	4.3
		Total in these spots	99.8	99.5	99.4	99.7	98.2	96.6
Glucose 1-C^{14}	Phosphate buffer 0.15 M	Pyruvic	98.4	92.8	91.1	85.1	78.5	59.2
		Lactic	1.2	2.6	2.8	2.6	2.3	2.4
		Citr Isocitr		—		—	—	—
		Succin Fumar	—				1.4	11.2
		Malic	—	—		1.2	3.6	9.8
		Total in these spots	99.6	95.4	93.9	88.9	85.8	81.6
Glucose 6-C^{14}	Tris buffer 0.15 M	Pyruvic	44.6	39.1	33.8	28.5	20.1	18.9
		Lactic	0.2	0.6	0.5	1.8	1.4	1.8
		Citr Isocitr	52.4	53.8	51.2	45.8	41.8	21.2
		Succin Fumar			4.3	7.1	10.6	17.7
		Malic				2.2	4.3	14.6
		Total in these spots	97.2	93.5	89.9	85.4	78.2	74.2

The experimental system was the same as that described in the legend of Table 1. In the phosphate experiments the tris buffer was replaced by a phosphate buffer of the same molarity.

Incorporation into organic acids

The comparatively large amount of the radioactivity of the total ethanol soluble fraction which is residing in non-phosphorylated compounds requires an analysis of the distribution in other metabolic intermediates. Among the compounds found in the aqueous ethanol extract the organic acids play an important part. In the second series of experiments these acids were accordingly isolated, and their radioactivity compared. One feature of the experimental system used is that OAA is decarboxylated to pyruvic acid. The pyruvic acid, on the other hand appears to be stable provided the ether extract is not evaporated completely to dryness until it has been carefully neutralized. Volatile acids are removed by the procedure. The activity distribution among spots corresponding to the tricarboxylic and dicarboxylic acids of the TCA cycle, and to pyruvic and lactic acids were determined and compared with the total activity of the ether soluble fraction.

Some activity distributions obtained in short time incubations with meningococcal cells have been recorded in Table 2. When considering

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A consideration of the succession in which the tricarboxylic and dicarboxylic acids are labelled from glucose 6-C^{14} may offer some information regarding the way the TCA cycle operates in meningococci Within the first few seconds after the addition of the labelled material considerable activity from the sixth carbon has already accumulated in the tricarboxylic acids and in pyruvate In contrast to this the dicarboxylic acids start to become labelled after a significant lag In the experiments recorded this lag lasted for approximately 20 seconds During the second half a minute however considerable radioactivity accumulated in these acids

In Table 2 an experiment has been included also which illustrates the effect of added exogenous P_i on the labelling of the TCA intermediates It is seen that glucose 1 C^{14} in the presence of 0 15 M P_i confers some radioactivity to the dicarboxylic acids during the second half a minute of incubation It does not however, label the tricarboxylic acids This shows that in cells which actively *catabolize* glucose the "secondary oxidation by way of the TCA cycle intermediates is significantly changed by the addition of P_i

Incorporation into amino acids of macromolecules

The material which was precipitated with hot aqueous ethanol was analysed mainly to explore the incubation time necessary for an incorporation of glucose carbon into macromolecules With the present knowledge of the biosynthetic pathways it was also considered likely that the way in which the amino acids were first labelled might offer some information regarding the pathways of glycolysis

From some data presented in Table 3 it will be seen that some amino acids in the hydrolysate become radioactive as early as 5 seconds after the addition of labelled glucose During the first minute of these incorporation experiments however the number was small of substances which were labelled Most of the activity of the hydrolysate was found in 7 spots Among these 5 were identified as amino acids as recorded in the table The other two spots which were not identified were labelled from glucose 1 C^{14} as well as from glucose 6-C^{14}

It is seen that alanine and valine were labelled from C-1 of glucose during the first 20 seconds of incubation These two amino acids on the whole account for the major part of the activity incorporated into the hydrolysate from this carbon during the first minute of incubation

When next we turn to the incorporation from C–6 we find a different picture. Already after the *first few seconds considerable activity was* found in the isoleucine and leucine spots. After 30 seconds this spot still accounted for 50 per cent of the total activity of the hydrolysate. At this time alanine and valine had also received radioactive carbon. During the second half minute of incubation aspartic acid and glutamic acid also had become labelled successively.

TABLE 3

Short-Time Incorporation of C–1 and C–6 from Specifically Labelled Glucose into some Amino Acids in Macromolecules of Neisseria meningitidis

Substrate	Amino acid analysed	Incubation period (seconds) Percentage distribution					
		5	10	15	20	30	60
Glucose 1-C^{14}	Alanine	64.8	63.2	64.5	62.3	60.8	53.7
	Valine	34.1	33.6	31.2	30.5	27.3	23.1
	Aspartic	–	–	–	–	–	–
	Glutamic	–	–	–	–	–	–
	Isoleucine-Leucine	–	–	–		–	–
	Total in these spots	98.9	96.8	95.7	92.8	88.1	76.8
Glucose 6-C^{14}	Alanine	–	11.4	20.2	21.2	19.3	16.3
	Valine	–	8.1	14.8	15.4	14.8	10.7
	Aspartic	–		0.2	0.5	2.2	4.1
	Glutamic	–		–	0.2	1.8	2.3
	Isoleucine-Leucine	97.2	78.2	58.7	55.7	49.1	37.8
	Total in these spots	97.2	97.7	93.9	92.6	87.2	71.2

The experimental system was the same as the one described in the legend of Table 1. The material which was precipitated with hot aqueous ethanol was hydrolyzed and the hydrolyzates analysed as described under methods.

DISCUSSION

The present investigation is concerned with the metabolism of meningococci which actively oxidize glucose. In order to obtain an orientation concerning the 'secondary' pathway operation as well as of the "primary" routes of glycolysis, conditions were similar to those *giving* maximal total oxidation of glucose rather than to those giving the maximal *rate of oxidation*. To obtain this the cells were limited on the supply of exogenous inorganic phosphate (*Jyssum, Borchgrevink & Jyssum* 1961, *Jyssum & Joner* 1962). The incorporation of C–1 and C–6 from glucose into phosphorylated compounds, organic acids and macromolecules corroborates the previous finding that the two atoms of glucose are *metabolized by different routes* (*Jyssum* 1962).

Analysis of the distribution among phosphorylated compounds indicates that the two carbons take different paths immediately after the

6-carbon stage has been passed. The heavy labelling of 6-phosphogluconic acid points out this substance as an important intermediate of both carbons. A kinetic study of the sequence in which C^{14} was detected in these compounds indicates that the C^{14} was first incorporated into sugar monophosphates, and then into 6-phosphogluconate. The labelling pattern of the phosphorylated compounds thus points to the operation of a pentose phosphate pathway or to an Entner-Doudoroff cleavage.

The observations concerning the labelling of organic acids provide further information. As a whole these analyses unequivocally demonstrate that at least a part of the C–1 is metabolized as far as to pyruvate. This finding requires some other major route of glycolysis than the pentose path. Since PEP is not labelled from C–1 an Entner-Doudoroff split in which C–1 reaches pyruvate directly from 6-phosphogluconate appears likely.

The labelling of organic acids furthermore provides information concerning the pathway of "secondary" oxidation. Under the conditions known to permit maximal "secondary" oxidation, *i.e.* with no added exogenous P_i (*Jyssum, Borchgrevink & Jyssum* 1961), C–1 does not confer radioactivity to the TCA intermediates, while C–6 does so rapidly. Hence, pathways from pyruvate, as well as the routes to pyruvate must be different for these two carbons of glucose under the conditions of the assay. This, of course, points to a different localization of the radioactivity from the two carbons in the pyruvate molecule, again an indication towards an operational Entner-Doudoroff pathway.

The effect of added exogenous P_i on the oxidation of glucose in meningococci has been found to be of a dual character. On the one hand the activity of the primary routes to pyruvate is very much enhanced (*Jyssum & Joner* 1962), on the other the total oxygen consumption is significantly reduced (*Jyssum, Borchgrevink & Jyssum* 1961). In the presence of P_i the oxidation proceeds rapidly to the acetate level while any further oxidation is very sluggish. This has been interpreted as an inhibition of the "secondary" oxidation of glucose. The present data are consistent with the assumption that the TCA reactions play a most important rôle as a "secondary" oxidative pathway in experiments without added P_i. The rapid labelling of PEP from C–6, and the equally rapid accumulation in the tricarboxylic acids may indicate that a major part of the C–6 labelled pyruvate is in fact the result of a decarboxylation of OAA synthetized from PEP by the PEP carboxylase (*Jyssum & Jyssum* 1962). The labelling of the tricarboxylic acids within seconds, as opposed to the dicarboxylic acids which are labelled only after 15–60 seconds may indicate that a "delay" exists in the operation of the TCA reactions, caused by a comparatively slow metabolization of the tricarboxylic acids.

Since exogenous P_i was assumed to block the "secondary" oxidation, the influence of added P_i on the labelling of the TCA cycle intermediates

was also explored The addition of 0 15 M P_i resulted in the labelling of the dicarboxylic acids from C-1, but without a simultaneous labelling of the tricarboxylic acids This is taken to indicate a synthesis of dicarboxylic acids from pyruvate by the malic enzyme (*Jyssum* 1960) Apparently the addition of P_i results in a block of the synthesis of dicarboxylic acids from tricarboxylic acids, and instead in a carboxylation of pyruvate via the Malic enzyme

Two mechanisms may be considered by which the concentration of P_i could exert its blocking effect on the "secondary" oxidation Previously we have suggested that this may be the result of a phosphate inhibition of the PEP carboxylase (*Jyssum & Jyssum* 1962) which would reduce the available OAA for a synthesis of tricarboxylic acids Another explanation would be that the blocking effect on the TCA cycle reactions is caused by a slow oxidation of TPNH This effect might be assumed to be pronounced in a microorganism without a transhydrogenase (*Jyssum* 1960) The two strictly TPN dependent enzyme systems of the TCA cycle, the isocitric dehydrogenase and the Malic enzyme (*Jyssum* 1960) may thus be "physiologically blocked" by such reactions which enhance the production of TPNH Since an operative "primary" glycolysis, and particularly a pentose phosphate route, tends to limit the available TPN (*Jyssum, Borchgrevink & Jyssum* 1961, *Jyssum & Jyssum*, to be published), an active "primary" glycolysis might in itself be assumed to depress the "secondary" oxidation by this mechanism

The quantity of pyruvic acid synthetized by a "primary" oxidation of glucose is increased more than 10 times as a consequence of the addition of 0 15 M P_i under otherwise identical conditions (*Jyssum & Joner* 1962) Such an active "primary" glycolysis results in the synthesis of large amounts of TPNH as well as of pyruvate, *i e* the conditions are favourable for a carboxylation of pyruvate to malate

The incorporation of activity from C-1 and C 6 into alanine and valine, and from C 6 into aspartic and glutamic acids agree well with the findings concerning the incorporation from the same carbons of glucose into the non nitrogenous precursors of these amino acids *pyruvic* acid, OAA and α-oxoglutaric acid

The heavy incorporation of radioactivity from C 6 into the isoleucine and leucine spots may be explained by a synthesis of isoleucine from α-oxobutyrate and the acetyl moiety of pyruvate (*Adelberg, Coughlin & Barratt* 1955) and leucine from α oxoisovalerate and acetate (*Abelson* 1954,*Abelson & Vogel* 1955 *McQuillen & Roberts* 1954) Since leucine and isoleucine are rapidly labelled solely from C 6 it appears likely that the activity originates from the acetyl part of pyruvic acid This explanation, of course, requires that the C 1 activity of the pyruvic acid must be located in the carboxyl carbon

The common carbon skeleton of valine and leucine α-oxovaleric acid, has been thought to arise from pyruvic acid and the acetyl moiety of another molecule of pyruvic acid with cleavage of the carbon skeleton

derived from the first molecule of pyruvic acid This takes place in such a way that the carboxyl and alpha carbons of pyruvic acid yield the carboxyl and alpha carbon atoms of α-oxovaleric acid (*Adelberg* 1955) The labelling of valine from C-1 and C-6, and of leucine and isoleucine from C-6 only, requires that the carbon C-1 from glucose is lost in the synthesis of α-oxoisocaproic acid from α-oxoisovaleric acid This agrees well with the general assumption that leucine is derived from the isobutyryl moiety of α-oxoisovaleric acid and from acetate (*Abelson & Vogel* 1955, *Abelson* 1954, *McQuillen & Roberts* 1954)

SUMMARY

The incorporation of radioactivity from glucose 1 C^{14} and glucose 6-C^{14} into phosphorylated compounds, organic acids and proteins has been investigated in short time experiments with suspensions of N meningitidis

The two carbons follow different pathways after the 6 carbon stage

Pyruvic acid is labelled from both carbons

Tricarboxylic acids are only labelled from 6 C^{14}

Incorporation into amino acids may agree with the hypothesis that the 1 C^{14} activity of pyruvic acid is located in the carboxyl carbon, while the 6 C^{14} activity is residing in the acetyl moiety of the molecule

These conclusions exclude the Embden-Meyerhof pathway as one of importance They also require the operation of some other pathway than the pentose phosphate route This pathway may be the Entner-Doudoroff cleavage

The data support the hypothesis that the TCA cycle reactions function as the main route of "secondary" glucose oxidation An inhibition of the TCA cycle reactions by inorganic phosphate during active glycolysis has been discussed

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FROM KAPTEIN W. WILHELMSEN OG FRUES BAKTERIOLOGISKE INSTITUTT,
OSLO UNIVERSITY AND RIKSHOSPITALET, OSLO
(HEAD: PROFESSOR S. DICK HENRIKSEN, M.D.)

DISSIMILATION OF C^{14} LABELLED GLUCOSE BY NEISSERIA MENINGITIDIS

3. *The Incorporation of 1-C^{14} and 6-C^{14} into pyruvate*

By

K. JYSSUM and P. E. JONER

Received 5.1.62

Previous studies with specifically labelled glucose indicate that the Embden-Meyerhof pathway contributes to a very small extent to the catabolism of glucose by cells of Neisseria meningitidis which are adapted to growth on a minimal medium. The labelling of various intermediates requires that some other pathway than the pentose phosphate route, probably the Entner-Doudoroff cleavage is also operative. These studies also indicate that the "secondary" oxidation of glucose proceeds via the tricarboxylic and dicarboxylic acids (*Jyssum* 1962 a, *Jyssum* 1962 b). In the present paper experiments are reported, based on the distribution and quantity of C^{14} in pyruvate isolated after catabolism of glucose-1-C^{14} and glucose-6-C^{14}. The study was undertaken to estimate the extent to which the oxidative pentose pathway and the Entner-Doudoroff route participate in the catabolism of glucose.

MATERIALS AND METHODS

The same materials and general methods as previously described were used (*Jyssum* 1962 a, *Jyssum* 1962 b).

Isolation of pyruvic acid. The keto acids were extracted from the reaction mixture as their 2,4-dinitrophenylhydrazones and separated by paper chromatography as previously described (*Jyssum* & *Jyssum* 1962).

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RESULTS

The accumulation of pyruvate was accomplished in these experiments by the use of a sodium arsenite block as described by *Entner* & *Doudoroff* (1952).

First, several experiments were performed with a view to exploring this system. An addition of P_i is apparently required in order to obtain

any considerable accumulation of pyruvic acid Some data which illustrate this effect are recorded in Table 1 In these experiments the addition of 300 μ moles P_i resulted in a maximal yield of pyruvate It is also seen that the addition of ATP or ADP could not relinquish this phosphate requirement under the conditions of the assay

TABLE 1

Influence of P_i and Preincubation on the Production of Pyruvate by Meningococci in the Presence of an Arsenite Block

Treatment of the suspension	Substance added	Quantity μmoles	Time of incubation Minutes	Pyruvate produced μmoles
Shaken at 37° for 30 min	–	–	30	0 19
	ADP	4	30	0 30
	ATP	4	30	0 22
	P_i	300	15	0 49
	P_i	300	30	1 16
	P_i	300	60	0 73
	P_i	300	120	0 33
No preincubation	–	–	30	0 21
	P_i	300	15	3 34
	P_i	300	30	3 14
	P_i	300	45	2 11
	P_i	300	60	1 83

The Warburg vessels contained a total volume of 3 2 ml The buffer was either 300 μmoles Tris or 300 μmoles P_i with pH 7 2 The substrate was 4 μmoles glucose which was tipped in from the sidearm at zero time The suspension was prepared in the buffer which was to be used in the actual experiment Analytical procedures and calculations were as described under methods The oxidation was blocked by 20 μmoles arsenite

The removal of P_i from meningococci metabolizing glucose in the presence of arsenite also resulted in a pronounced reduction of the oxygen consumption This influence on the oxidation is in remarkable contrast to the result of the removal of P_i in a similar system without the arsenite block In such a system the initial rate of oxidation is very moderately reduced, the total oxygen consumption, however, is rather enhanced (*Jyssum, Borchgrevink & Jyssum* 1961) The CO_2 concentration seems to have no influence on the accumulation of pyruvate in these experiments, since with *KOH* in the center well the accumulation was the same as in experiments without

Apparently the quantity recovered depends to some extent upon the treatment of the cell suspension It will be seen from the table that a suspension which had been shaken at 37° in order to reduce the endogenous respiration gave approximately half as much pyruvic acid as did fresh suspensions It may be suggested that the increased quantity of pyruvic acid in fresh suspensions originates from substrate carbon accumulated in the suspension That such carbon does not contribute to

the pyruvate as the major factor may be deduced from the radioactivity of the pyruvate accumulated from labelled glucose. These data indicate that endogenous carbon contributes to no more than 10 per cent of the extra carbon accumulated as pyruvic acid in fresh suspensions.

The production of pyruvic acid is far from being quantitative. Large amounts of carbon escaped the arsenite block and appeared as CO_2 and acetate.

Thus, a more prolonged incubation, i.e. more than 30 minutes always resulted in a steady decrease in the recovery of pyruvate. Between 30 and 120 minutes after the start of the experiment the quantity accumulated fell to less than 50 per cent. Under the conditions of the assay glucose is obviously very rapidly metabolized to pyruvate, while this substance is later slowly "leaking" through the arsenite block. An increased concentration of arsenite did not change significantly these results.

TABLE 2

Incorporation of Radioactivity from Specifically Labelled Glucose into Pyruvic Acid in the Presence of an Arsenite Block

Substrate µmoles	Treatment of the suspension	Pyruvic acid trapped µmoles	Per cent of total activity	SA CPM µmole 10^3	Corrected RSA per cent	Per cent in carboxyl carbon
Glucose 1 C^{14} 1.71 × 10^5 CPM	No preincubation	3.58	19.9	9.75		
		3.43	19.2	9.55		
		3.12	19.5	10.70		
		3.06	18.3	10.20		
Glucose 1 C^{14} 1.58 × 10^5 CPM	No preincubation	3.43	16.4	7.56	78.5	
		3.56	18.3	8.14	84.5	
		2.92	11.1	5.97	67.0	100.5
	Shaken at 37°	1.42	5.5	7.14	74.3	
		1.41	6.9	7.78	80.8	99.8
Glucose 6 C^{14} 1.13 × 10^5 CPM	No preincubation	2.58	16.7	7.30	114.7	0.97
		2.88	20.8	8.18	121.7	
	Shaken at 37°	1.59	9.3	6.64	124.0	0.86
		1.69	9.6	6.44	120.1	
Glucose 1 C^{14} 1.57 × 10^5 CPM	No preincubation No P_1 added	0.22		4.5	87.5	103.2
Glucose 6 C^{14} 1.13 × 10 CPM	No preincubation No P_1 added	0.21		5.9	116.2	5.3

The experimental system was the same as the one described in the legend of Table 1. RSA was corrected for endogenous production of pyruvate by means of parallel experiments with glucose 1 C^{14}. The experiments represent different cell populations.

On the basis of these findings it was possible to study the radioactivity of pyruvic acid accumulated after incubation periods not exceeding 30 minutes

In Table 2 data from some representative experiments are summarized In these experiments the influence of P_i, and of the "starving of the meningococci, on the relative specific activity and radioactivity distribution in pyruvic acid has also been explored

Pyruvate arising exclusively via the Entner-Doudoroff and the Embden-Meyerhof pathway from either glucose 1–C^{14} or glucose 6–C^{14} would have a corrected relative specific activity (RSA) of 100 (*Lewis, Blumenthal, Weinrach & Weinhouse* 1955) In several experiments with meningococci an average of 78 per cent was found The scattering of RSA in separate experiments between the lower value of 67, and the top value of 87 5 per cent is more than might be expected from the method according to the parallels, and may be taken to indicate a true variation between different cell populations This variation could not be correlated with the "starving" of the cells, nor with a presence or absence of P_i.

The corrected RSA from glucose 1–C^{14} indicates that only (on an average) 78 per cent of the labelled pyruvate carbon could have arisen from either of the two processes mentioned Since more than 99 8 per cent of the labelled pyruvate carbon was in the carboxyl group it may be assumed that no more than $0\ 02 \times 78 = 0\ 15$ per cent arose via the Embden-Meyerhof pathway These data correspond well with the analyses of specific activity in CO_2 and acetate (*Jyssum* 1962 a), and corroborate the finding that the Embden-Meyerhof pathway plays an insignificant rôle in the metabolism of glucose in resting cells of N meningitidis (*Jyssum 1962 b*)

If all the pyruvate was produced via the Entner-Doudoroff pathway the corrected RSA from glucose 6 C^{14} could not have been more than 100 The observed average value of 119 5 per cent (scattered between 114 7 and 124 per cent in individual experiments) indicates a preferential utilization of carbon six for pyruvate formation This points out the pentose pathway as a major process for pyruvate formation The RSA from glucose 6 C^{14} is as a whole not far from the one which might be expected on a basis of 78 per cent Entner-Doudoroff and 22 per cent oxidative pentose phosphate pathway participation The scattering of the RSA values in the individual experiments corroborates the assumption of a small variation in the relative pathway participation between the individual cell populations

Pyruvate labelled from glucose 6 C^{14} via the Entner-Doudoroff pathway should have no activity in the carboxyl group Pyruvate synthetized via the oxidative pentose pathway might also be expected to be without activity in this carbon, even if a migration of glucose 6–C to glucose 1 C cannot be excluded (*Lewis Blumenthal, Weinrach & Weinhouse* 1955) The finding of less than 1 per cent of the activity from glucose 6 C^{14} in the carboxyl carbon of pyruvic acid in several experiments is thus in

agreement with the previous assumptions The somewhat higher percentage found in the experiments without added P_i may, if significant, indicate a more pronounced migration under these conditions

DISCUSSION

By means of an arsenite block more definite information may be obtained concerning the respective participation of the pentose path and the Entner-Doudoroff route of glucose catabolism

Optimal conditions for the recovery of pyruvate in meningococci required the presence of inorganic phosphate in the concentration 0 1 to 0 15 M The removal of P_i resulted in a decrease in the recovery to less than 10 per cent under otherwise identical conditions

These results may be taken as an indication that the P_i dependent GA-3-P dehydrogenase acts as an important rate limiting factor in the primary glycolysis in meningococci Since the oxidation in cells without an arsenite block is not affected to the same extent by the removal of P_i it may also be assumed that the important factor is the energy released by the GA-3-P dehydrogenase Accordingly, when the rate of the reaction is decreased by a limited supply of P_i the ATP level cannot be maintained sufficiently high to permit the operation of the glucokinase in the presence of arsenite The requirement for P_i, however, could not be substituted by ATP This may indicate that the high concentration of P_i, besides the direct function as available substrate for phosphorylations, may function as an inhibitor for such reactions which drain the pools of energy Such reactions may be the PEP carboxylase and the phosphatases (*Jyssum & Jyssum* 1962)

Whatever the mechanism, however, it is evident that the concentration of P_i has a remarkable influence on the rate of the primary glycolysis in meningococci

Clearly, we are left with the task of explaining a double effect of P_i on the total glycolysis in meningococci On the one hand the enhancing effect on the primary oxidation to pyruvic acid, and on the other the depression of a more complete oxidation below the acetate level (*Jyssum, Borchgrevink & Jyssum* 1961)

In a previous communication we have suggested mechanisms by which the presence of P_i may be thought to inhibit a "secondary" oxidation via the tricarboxylic and dicarboxylic acids (*Jyssum 1962 b*)

A fall in the endogenous respiration in meningococcal suspensions coincided with a fall in the quantity of pyruvate recovered in the presence of an arsenite block The data on radioactivity demonstrate that the major part of the increased accumulation in fresh cells with high endogenous activity originated from the substrate glucose added, and not from endogenous carbon sources Thus, the increased accumulation of pyruvate seems to occur "in the heat of the endogenous respiration"

In meningococci the addition of arsenite did not completely block a

further metabolization of pyruvate During prolonged incubation the pool of accumulated pyruvate decreased steadily Since the oxidation to pyruvate occurred at a very rapid rate in the presence of P_1, and much faster than the further metabolization by "leakage", maximal accumulation of pyruvic acid was found as early as 15 to 30 minutes after the addition of the substrate glucose The finding of a "leakage" of the arsenite block has been reported in Pseudomonas fluorescens (*Lewis, Blumenthal, Weinrach & Weinhouse* 1955) The same block seems to yield a quantitative production of pyruvate in Pseudomonas saccharophila (*Entner & Doudoroff* 1952)

The analyses of the localization of the radioactivity in pyruvate which had been labelled from the first and the sixth carbon of glucose agree well with the previous findings in eliminating the Embden-Meyerhof pathway as a quantitatively significant mechanism of glycolysis in *N meningitidis* (*Jyssum* 1962 a, *Jyssum* 1962 b) It may be concluded that this pathway contributes to no more than approximately 0 3 per cent of the pyruvate produced

The heavy labelling of the carboxyl group in pyruvic acid from glucose 1-C^{14} corroborates the previous assumption that this micro organism uses the Entner-Doudoroff pathway as a major route of glucose catabolism (*Jyssum* 1962 b) The tracer data indicate that on an average 78 per cent of the glucose is metabolized by this route while the rest is metabolized via the pentose phosphate reactions The respective pathway participation of these two routes seems to change from one cell population to the other in the way that the Entner-Doudoroff split has been found to contribute between 67 per cent and 87 per cent in individual experiments The variation in the relative activities of the two pathways could neither be correlated with the influence of P_1 on the primary glycolysis, nor with the effect of a reduced endogenous respiration

It appears likely that the TPN/TPNH quotient is a major factor in the activity distribution between the two pathways This is emphasized by the pronounced influence of the TPN concentration upon the activity of the 6 phosphogluconate dehydrogenase in meningococci (*Jyssum & Jyssum*, to be published)

There is some indication that the glutamic dehydrogenase system may serve as a transhydrogenase in meningococci which are adapted to growth with glucose as their sole carbon source (*Jyssum & Borchgrevink* 1960) Such an activity might be expected to be influenced by a change in the activity of the TCA cycle reactions, *i e* indirectly by a removal of the arsenite block

As a consequence of these considerations the present evaluation of the relative pathway participation of the two major routes of glucose catabolism should not directly be extended to meningococci which catabolize glucose without an arsenite block

The studies performed by tracer technique have revealed in N me-

ningitidis a pattern of glucose catabolism which previously has been found typical of the pseudomonads In contrast to these bacteria the meningococci are obligatory human parasites and potential pathogens However, the two kinds of microbes have one thing in common· they are both equipped with metabolic systems based on aerobic respiration

SUMMARY

Pyruvate produced from labelled glucose has been examined in suspensions of Neisseria meningitidis in which the oxidation was blocked with arsenite Relative specific activities (RSA) have been calculated and corrected for endogenous metabolism The localization of the radioactivity in the pyruvate molecule was explored

The presence of P_i enhanced the production of pyruvate

Pyruvate from glucose 1 C^{14} had a moderate, corrected RSA (average 78 per cent) More than 99 5 per cent of the activity was located in the carboxyl carbon

Pyruvate from glucose 6 C^{14} had a high corrected RSA (average 119 per cent) A very small part of this activity was located in the carboxyl carbon

Calculations from the experimental data are taken to indicate:

1 The Embden-Meyerhof process contributes to no more than 0 3 per cent of the pyruvate

2 The Entner-Doudoroff cleavage does always synthetize the major part of pyruvate Between 67 and 87 per cent of the glucose molecules have been found to be catabolized via this route with variations between individual cell populations

3 The pentose phosphate pathway accounts for the remaining part of glucose which is broken down to pyruvate

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ATYPICAL STRAINS OF CLOSTRIDIUM PERFRINGENS FROM SWINE

By

INGMAR MÅNSSON and LOUIS DS. SMITH

Received 11.62

When swine were placed on a diet high in protein, the microbial flora of the intestine was found to change markedly (*Månsson & Olsson* 1961 a, b, c). Especially noticeable was the increase in the number of clostridia, particularly one resembling *Clostridium perfringens* which increased from about 250 per gram of faeces of swine on a normal diet to about 1,000,000 per gram in swine on a high-protein diet. Many strains of this organism resembled *C. perfringens* but were not typical of this species in one or more cultural characteristics, particularly in proteolytic properties and ability to ferment salicin.

The present communication presents the results of a study of 35 strains isolated from this source that were more than ordinarily proteolytic.

MATERIAL AND METHODS

The strains from swine were isolated from faeces or intestinal content on horse or beef blood agar plates incubated in an atmosphere of hydrogen. The faeces samples were collected in such a way that contamination with soil etc. was avoided.

Control of the Purity of the Strains

Although all of the strains were in apparently pure culture, they were replated several times before use in the experiments described in this paper. Because of the necessity of being as nearly certain as possible of the purity of our cultures, a good deal of attention was given to this point. The strains were grown in semi-solid liver infusion medium for two days at 35° C to detect any possible motile contaminants. They were then transferred to the surface of bovine blood agar plates which were incubated for 2 days in Brewer jars under an atmosphere of hydrogen. These plates were then examined with the aid of a hand lens or a dissection microscope and well isolated colonies were selected. A portion of each colony was Gram stained and examined microscopically. The remainder of the colony was transferred to a tube of broth from which another blood agar plate was streaked. After anaerobic incubation overnight these plates were again carefully examined and well isolated colonies were suspended in broth from which dilutions in tubes of egg yolk agar (*McClung & Toabe* 1947) and pour plates were made. After anaerobic incubation of these plates, isolated colonies were carefully selected and portions were Gram stained and examined microscopically. The remainder of each colony was then transferred to chopped meat medium and to brain medium for preservation. At no time was there evidence of more than one morphological or cultural type of organism in

any of these strains Purity of the cultures was verified from time to time during the course of these experiments especially those in milk medium which showed evidence of digestion of the casein and in fermentation broth in which salicin had been fermented

Biochemical Tests

was no difference in the performance of the bacteria on these solutions of salicin Thioglycollate broth was used as a basal fermentation medium to which the sterile carbohydrate solutions were added A test for indol production was made by growing the strains in fluid thioglycollate medium Kovac s reagent was used as an indicator A test for

following

1 g agar

as an indi

to tubed whole milk before autoclaving

The proteolytic capacity of the strains was tested in gelatin coagulated serum and casein Thiogel (Baltimore Biological Laboratories) was used for gelatin liquefaction Coagulated serum medium was made by adding 10 per cent bovine serum to nutrient broth and sterilizing by autoclaving and also by adding two volumes of sterile bovine serum to one volume of sterile glucose broth and coagulating in flowing steam Casein agar was made by dissolving one per cent casein in blood agar base and adjusting the pH approximately to neutrality before autoclaving On this medium colonies of protolytic strains were surrounded by barely visible haloes Such digestion of the casein became much more apparent when the plates were flooded with a saturated solution of ammonium sulphate or with 10 per cent hydrochloric acid

Toxin Production

The ability to prod ce kapp ions for five hours 0 5 ml portions of muscle at the site presence of pulpy muscle lacking its normal collagenous framework was taken as evidence that the strain in question produces kappa toxin *in vivo*

The ability to produce proteolytic enzyme inhibited by kappa or lambda antitoxin was determined by the method of *Oakley et al* (1948) The strains were grown for 15 hours in beef infusion broth to which 3 per *cent peptone* 0 5 per cent yeast extract and 0 5 per cent sodium chloride had been added The cultures were centrifuged and 1 0 ml of the supernatant fluid was poured over columns of one per cent agar containing 0 4 per cent gelatin pH 7 0 in 6 × 50 mm tubes with or without prior treatment for 30 minutes with 10 units of kappa or lambda antitoxin per ml The tubes of gelatin agar and neutralized or un neutralized culture fluid were incubated overnight at 35° C The overlying fluid was then poured off and was replaced by a solution of 20 per cent hydrochloric acid containing 15 per cent mercuric chloride and the tubes were allowed to stand at room temperature for two hours before examination

The production of major lethal toxins was investigated by growing the strains overnight in chopped meat medium with four per cent trypticase and one per cent soluble starch centrifuging and adjusting the supernatant fluid to neutrality This was divided into two portions adding to one of these 0 25 per cent trypsin upon which it was incubated for one hour at 35° C. The other portion was left untreated Four tenths ml portions of the untreated and the trypsin treated material from each culture were inoculated intraperitoneally into mice, using two animals for each portion Culture filtrates which were sufficiently toxic to kill mice were treated with 10 units of alpha antitoxin per ml left for one hour at room temperature, and inoculated into mice

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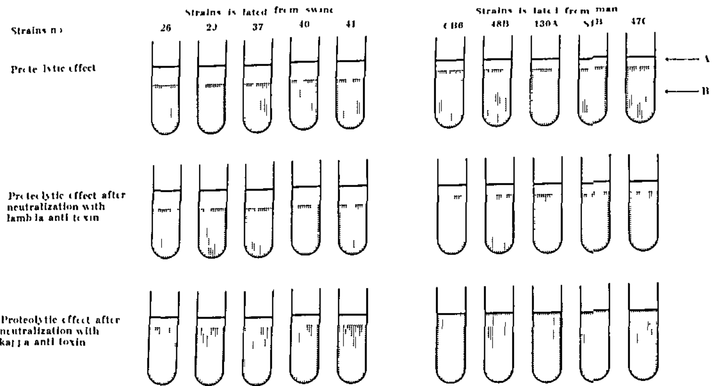


Figure 1 The proteolytic effect in gelatin agar — The figure demonstrates stage 2 according to *Oakley et al* (1948) i e the not precipitated (A) and the precipitated (B) zones of the gelatin column after the toxin and antitoxin solutions were replaced by hydrochloric acid and mercuric chloride For the sake of surveyability the blank of small dimension which is always obtained in the upper part of the column and the not precipitated gelatin in the bottom are disregarded

RESULTS

Morphology, motility and sporulation ability

All of the strains were composed of short, plump, Gram-positive rods occurring singly and in pairs, sometimes in chains of four. No evidence of motility in semisolid medium was seen in any of the strains. Several attempts were made to induce sporulation in these strains by heavy inoculation into *Ellner's* (1956) medium. Spores were seen after this treatment in only one of the strains and in this strain, only about three per cent of the cells were sporulating. The spores were oval in shape, subterminal in position, and swelled the rods. Heating of cultures at 75° C for a few minutes would kill these; apparently, heat-resistant spores were not formed by these strains under these conditions.

Growth on agar

Colonies on blood agar plates were three to seven mm in diameter, circular, slightly raised, with opaque centers. Most of the strains had regular, entire margins but a few had flattish irregular margins such as are not uncommonly found with strains of *C. perfringens* which have been in culture for some time. All of the colonies were immediately surrounded by zones of complete haemolysis presumably caused by theta toxin and also outer zones of partial haemolysis and discoloration presumably caused by alpha toxin. Colonies on *McClung & Toabe's* (1947) agar plates were somewhat smaller than those on blood agar plates and were surrounded by zones of precipitation indicative of lecithinase (alpha toxin) activity.

Biochemical properties

All of the strains fermented glucose, maltose, lactose, and sucrose, reduced nitrate to nitrite, caused stormy fermentation in iron milk. Digestion and blackening of iron milk varied from strain to strain. This variation in activity may have been caused by the irregular effect or production of acid by the fermentation of the lactose of the milk. This fermentation of lactose may also have been the reason for the comparatively poor digestion of casein observed on *Willis & Hobb's* (1959) medium, in contrast to the readily apparent digestion shown on casein agar. There was no marked digestion of chopped meat medium. No strain formed indol.

Sixteen of the thirty-five strains were active fermenters of salicin. There was no obvious correlation between digestion and blackening of iron-milk and the fermentation of salicin. Of the 16 strains which digested and blackened iron-milk, 7 fermented salicin and 9 did not; of the 19 strains which did not obviously digest casein in milk, 9 fermented salicin and 10 did not.

inulin was once used as the basis for fermentative types but because ability or the lack of it to ferment these sugars was not correlated with any other characteristic, these fermentative types have been ignored The other cultural characteristics which have been the subject of varying bacteriologic opinion are the ability to digest coagulated serum and the ability to ferment salicin

Some of the earlier workers were inclined to ascribe considerable proteolytic ability to this organism, although *Robertson* (1929) considered it unable to digest coagulated proteins She pointed out that apparently proteolytic strains of *C perfringens* were often mixtures of this organism with *Clostridium sporogenes* with the latter being held markedly in subjection, a phenomenon often overlooked by other workers Nevertheless, *Wilsdon* (1931) reported all of 11 strains of type B to digest coagulated serum as well as 14 of 25 strains of type A More recently, *Henriksen* (1937) found none of 24 strains of *C perfringens* to digest coagulated serum *Prevot* (1948) described type B strains but not type A strains as digesting this substrate as did *Smith* (1955) *Breed et al* (1957) have stated that *C perfringens* is unable to digest coagulated serum and *Willis* (1960) considering it as having no marked proteolytic activity

Fermentation of salicin has also been variously reported *Robertson* (1929) found it not to be fermented *Wilsdon* (1931) indicated that some strains fermented salicin after prolonged incubation, *Henriksen* (1937) found one of 24 strains to ferment it *Prevot* considered this sugar as rarely fermented, as did *Smith* (1955), and *Breed et al* (1957) There have been no reports of any tendency toward association between salicin fermentation and toxigenic types, such as Wilsdon encountered with type B strains and digestion of coagulated serum

None of the proteolytic strains from swine studied by us could be classified as type B strains Indeed, their production of alpha theta, and kappa toxins and their inability to produce perceptible amounts of beta gamma delta epsilon, lambda or iota toxins place them clearly in type A Their production of an enzyme not usually produced by type A strains of *C perfringens* which allows them to digest coagulated serum would not remove them from type A

It is evident from the study of these strains that *C perfringens* is variable so far as these characteristics are concerned Neverthele[illegible] of [illegible]
tu[illegible]
pr[illegible] [illegible]ong to the species *Clostridium perfringens* and should be placed in type A

SUMMARY

Thirty-five markedly proteolytic strains of *C perfringens* isolated from swine on a diet high in protein were studied culturally and toxi-

All strains liquefied gelatin, digested coagulated serum both in autoclaved serum-broth and as modified Loeffler's medium, and digested casein in casein-agar plates

Toxin production

All 35 strains were investigated for the production of lethal toxin by the inoculation of mice The culture fluids of three strains killed one of the two mice into which each of these had been inoculated One strain produced sufficient toxin to kill both of the inoculated mice The culture fluids of the remaining strains killed no mice When 10 units of *C perfringens* alpha antitoxin were added per ml of culture fluid and the mixture allowed to stand at room temperature for one hour before inoculation into mice, no toxicity was evident It appears thus that the only lethal toxin produced by these strains was alpha toxin

Toxinproduction compared with known type A strains

Five of the strains isolated from swine were chosen for a more detailed comparison with five classical type A strains which had been isolated from cases of gas gangrene in man in World War II by *Smith & George* (1946) and had been preserved in tubes of dry soil since that time Because the strains from swine liquefied gelatin it was concluded that they were producing a proteolytic enzyme, probably either kappa or lambda toxin Accordingly, these ten strains were tested by the method of *Oakley et al* (1948) for the differentiation of kappa and lambda toxins The results, shown in Figure 1, indicate that the strains from gas gangrene produced kappa toxin, the proteolytic action of their culture fluids was inhibited by kappa but not by lambda antitoxin The strains isolated from swine had a proteolytic effect which was not inhibited by lambda antitoxin and only partially by kappa antitoxin Apparently the strains produced a proteolytic factor that was neither kappa nor lambda toxins This factor will be subjected to further studies

Kappa antitoxin caused a certain inhibition of the proteolytic effect of the swine strains Consequently, we endeavoured to demonstrate the production of kappa toxin by inoculating these intramuscularly into guinea pigs, as we did the gas gangrene strains After the death of the animals, the sites of inoculation were examined Marked pulping of the muscle was found with all strains and it was concluded that kappa toxin was produced by the strains isolated from swine as well as a proteolytic factor acting on coagulated serum and gelatin

DISCUSSION

C perfringens is comparatively uniform in its cultural characteristics Some variation has been found, however, in certain characteristics The ability of some strains, but not of all, to ferment glycerol or

FROM STATENS SERUMINSTITUT (INTERNATIONAL SALMONELLA CENTER)
COPENHAGEN

SUPPLEMENT TO THE KAUFFMANN-WHITE-SCHEME (V)

By

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Received 10.1.62

This paper is the 1. supplement to the *Kauffmann-White-Schema* given in the book "*Die Bakteriologie der Salmonella-Species*" (*Kauffmann 1*) and the 5. supplement to the review "*Das Kauffmann-White-Schema*" (*Kauffmann 2*). It contains *69 Salmonella species* recognized during 1961. From these 69 *species* 47 belong to *sub-genus* I and 22 to *sub genus* II (*Kauffmann 3*). The *species* of *sub-genus* II are indicated by an asterisk (*).

Deviating biochemical results, not given in Table 2, were obtained in the following *species*:

S. kivu, *S. hueningen*, *S. bergedorf*, one culture of *S. bahrenfeld* and *S. vleuten* were indole-positive.

S. kivu, *S. hueningen* and *S. vleuten* fermented salicin after 1–2 days.

S. kivu was very bad smelling.

S. soesterberg, *S. houten*, *S. tuindorp*, *S. wassenaar* and *S. bonair* were KCN-positive. *S. soesterberg* fermented salicin after 2 days, *S. houten* after 3 days, *S. wassenaar* after 1–2 days and *S. bonair* after 1 day.

TABLE 1
Some Deviating Biochemical Results

Species	Dulcitol	Gelatin	Indole	Salicin	KCN
S. kivu		—	+	+1 2	—
S. hueningen	+	+	+	+1	—
S. soesterberg		+	—	+2	+
S. houten	—	+	—	+3	+
S. tuindorp	—	+	—	—	+
S. vleuten	+	—	+	+1 2	—
S. wassenaar	—	+	—	+1 2	+
S. bonair		+	—	+1	+

The other biochemical res...
According to the definiti...
types (Kauffmann 4) ...
or not ar... ...lmonella ...

genically Except for their ability to digest coagulated serum and for sixteen of them their ability to ferment salicin, culturally they were typical strains Digestion of coagulated serum was apparently brought about by an extracellular enzyme which was not identical, serologically with either kappa or lambda toxins These strains did produce alpha kappa, and theta toxins but not beta, gamma, delta epsilon lambda or iota toxins and, consequently, belong in type A

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FROM STATENS SERUMINSTITUT (INTERNATIONAL SALMONELLA CENTER) COPENHAGEN

SUPPLEMENT TO THE KAUFFMANN-WHITE-SCHEME (V)

By

F KAUFFMANN

Received 10 1 62

This paper is the 1 supplement to the *Kauffmann-White Schema* given in the book "*Die Bakteriologie der Salmonella-Species*" (*Kauffmann 1*) and the 5 supplement to the review "*Das Kauffmann White Schema'* (*Kauffmann 2*) It contains *69 Salmonella species* recognized during 1961 From these 69 *species* 47 belong to *sub-genus* I and 22 to *sub genus* II (*Kauffmann 3*) The *species* of *sub-genus* II are indicated by an asterisk (*)

Deviating biochemical results, not given in Table 2, were obtained in the following *species*

S kiwu, S hueningen, S bergedorf, one culture of *S bahrenfeld* and *S vleuten* were indole-positive

S kiwu, S hueningen and *S vleuten* fermented salicin after 1–2 days

S kiwu was very bad smelling

S soesterberg, S houten, S tuindorp, S wassenaar and *S bonair* were KCN-positive *S soesterberg* fermented salicin after 2 days, *S houten* after 3 days *S wassenaar* after 1–2 days and *S bonair* after 1 day

TABLE 1

Some Deviating Biochemical Results

Species	Dulcitol	Gelatin	Indole	Salicin	KCN
S kiwu		—	+	+1 2	—
S hueningen	+	+	+	+1	—
S soesterberg		+		+2	+
S houten	—	+	—	+3	+
S tuindorp	—	+	—	—	+
S vleuten	+	—	+	+1 2	—
S wassenaar	—	+	—	+1 2	+
S bonair	—	+	—	+1	+

The other biochemical results are given in Tabl

According the inf [illegible]

types [illegible] entative phage

[illegible] ed as species"

TABLE 2

Species	Ara	Dul	Ino	Rha	Tre	Xyl	Cly	H_2S	Gel	d	l	l	Cit	Muc	Mal
S *agona* = 4 12 f g s —	+	+	+	+	+	+	+ +	+	—	+	+	+[2]	+	+	—
Salmonella = 4 5 12 g (p) 1 7	+	+	—	+	+	+	+ +	+	+	+	—	—	+	+	—
S *neumuenster* = 1 4 12 27 k 1 6	+	+	—	+	+	+	+ +	+	—	+	+	+[2]	+	+	—
S *tafo* = 1 4 12 27 z_{35} 1 7	+	+	--	+	+	+	d	+	—	+	d	+-[4]	+	+	—
Salmonella = 4 12 z_{36} —	+	+	+	+	+	+	+ +	+	—	+	+	—	+[2]	+	—
•S *makumira* = 4 12 e n x 1 7	+	+	—	+	+	+	+ +	+	+	×	—	—	+[2]	+	+
S *kuru* = 6 7 d 1 6	+	—	—	+	+	+	+ +	+	—	×	+	—	+	+	—
S *norton* = 6 7 i l w	+	+	+	+	+	+	+ +	+	—	+	+	+[2]	+	+	—
Salmonella = 6 8 c 1 2	+	+	+	+	+	+	+ +	+	—	+	+	+-	+	+	—
S *chincol* = 6 8 g m s e n x	+	+	—	+	+	+	+ +	+	—	+	—	—	+	+	—
S *nijpra* = 6 8 z_{10} z_6	+	+	+	+	+	+	+ +	+	—	+	+	+	+	+	—
•S *hueningen* = 9 12 z z_{39}	+	+	—	+	+	+	+ +	+	+	—	—	—	+[2]	+	+
•S *lundby* = (9) 46 b e n x	+	+	—	+	+	+	+ +	+	+	×	—	—	+[2]	+	+
S *bamboye* = (9) 46 b l w	+	+	—	+	+	+	+ +	+	—	+	+	+[2]	+	+	—
S *bergedorf* = (9) 46 e h 1 2	+	+	—	+	+	+	+ +	+	—	+	+	+	+	+	—
S *wernigerode* = (9) 46 f g —	+	+		+	+	+	+ +	+	—	+	×	—	+	+	—
Salmonella = (9) 46 g s t —	+	+	-	+	+	+	+ +	+	—	—	+	+	+	+	—
S *benfica* = 3 10 b e n x	+	+	+	+	+	+	+ +	+	—	+	+	+	+	+	—
•S *islington* = 3 10 g t —	+	+		+	+	+	+ +	+	+	×	—	—	+[2]	+[1 2]	+
S *ohlstedt* = 3 10 y e n x	+	+	-	+	+	+	+ +	+	—	+	—	—	+	+	—
•S *finchley* = 3 10 z e n x	+	+	-	+	+	+	+ +	+	+	×	—	—	+[2]	+	+
•S *chudleigh* = 3 10 e n x 1 7	+	+	—	+	+	+	+ +	+	+	—	—	—	+[3 4]	+	+
S *tournai* = 3 15 y z_6	+	+		+	+	+	+ +	+	—	+	+	+[2]	+	+	—
S *bedford* = 1 3 19 l z_{13} z_{28} e n z_{15}	+	+	+	+	+	+	+ +	+	—	+	+	+[2]	+	+	—
•S *huila* = 11 l z_{28} e n x	+	+	—	+	+	+	+ +	+	+	×	—	—	+-	+	+
S *volta* = 11 r l z_{13} z_{28}	+	+	+	+	+	+	+ +	+	—	+	×	—	+-	+	—
S *mim* = 13 22 a 1 6	+	+	—	+	+	+	+ +	+	—	+	×	—	+[2]	+	—
S *mampong* = 13 22 z_{35} 1 6	+	+	-	+	+	+	+ +	+	—	+	×	—	+[2]	+	—
Salmonella = 13 23 i e n z_{15}	+	+-	+	+	+	+	+ +	+	—	+	—	—	+[2]	+	—
•S *katesgrove* = 1 13 23 m t 1 5	+	+	-	+	+	+	+ +	+	+	×	—	—	+[2]	+	+
S *linton* = 13 23 r e n z_{15}	+	+		+	+	+	+ +	+	—	+	×	—	+	+	—
S *bahrenfeld* = 6 14 24 e h 1 5	+	+	—	+	+	+	+ +	+	—	+	×	—	+	+	—
S *fischerkietz* = 1 6 14 25 y e n x	+	+	—	+	+	+	+ +	+	—	+	×	×	+	+	—
S *soahanina* = 6 14 24 z e n x	+	+	—	+	+	+	+ +	+	—	+	×	—	+	+	—
S *wi* = 16 b 1 5	+	+	+	+	+	+	+ +	+	—	+	+	+	+	+	—
•S *elsiesrivier* = 16 z_{42} 1 6	+	+	—	+	+	+	+ +	+	+	+[2]			+	+	+
S *zeist* = 18 z_{10} z_6	+	+[4]		+	+	+	+ +	+	+	×				+	

S x esterberg — 41 z_4 z_{23}	+			+	+	+		+	+	×			+°	−	—
*S manlsbek — 27 z_1 z_6	+	+		+	+	+	+ +	+	+	$+^{3}$	—	—	$+^{2}$	+	+
S techiman — 28 c z_6	+	+	+	+	+	+	+ +	+	—	+	+	$+^{2}$	+	+	—
S mocamedes — 28 d e n x	+	+	—	+	+	+	+ +	+	—	+	$+^{2}$	$+^{2}$	+	+	—
S altmstean — 28 k 1 6	+	$+^{2}$		+	+	+	+ +	+	—	+	×	—	$+^{2}$	+	—
*S ceres — 28 z z_{39}	+	+	+	+	+	+	+ +	+	+	—	—	—	+°	+	+
S ehlendorf — 30 a 1 5	+	+	+	+	+	+	+ +	+		+	×	—	+	+	—
S fujicinte — 38 g t	+	+		+	+	+	+ +	+	+	×	—		$+^{2}$	+	—
*S suares — 1 40 c e n x z_1	+	+		+	+	+	+ +	+	+	—	—	—	+	+	+
*S alsterdorf — 1 40 g m t	+	+		+	+	+	+ +	+	+	×	—	—	$+^{2}$	×	+
S nrleanes — 40 z z_6	+	+	—	+	+	+	+ +	+	—	+	×	×	+	+	—
*S fanfrin — 1 40 z_{15} e n x z_{15}	+	+		+	+	+	+ +	+	+	—	—	—	$+^{2}$	+	+
S vietnam — 41 b	+	+	+	+	+	+	+ +	+	—	+	+	$+^{2}$	+	+	—
S equsi — 41 d	+	+		+	+	+	+ +	+	—	+	+	+	+	+	—
S eguitoa — 1 42 b z_6	+	+	+	+	+	+	+ +	+	—	+	+	—	+	+	—
*S portbech — 42 l v e n x z_{15}	+	+		+	+	+	+ +	+	+	×		-	$+^{2}$	+	+
S houten — 43 z_4 z 3	+			+	+	+	—	+	+	×	—	—	$+^{2}$	-	—
S tuindorp — 43 z_4 z_{32} —	+	—		+	+	+	—	+	+	×	—	—	$+^{2}$	—	—
S fischerstrasse — 44 d e n z_{15}	+	+	—	+	+	+	+ +	+		+	+		$+^{2}$	+	—
S vleuten — 44 f g —	+	+		+	+	+	+ +	+		+	×	$+^{3,4}$	+	+	—
*S bilthoven — 47 a —	+	+		+	+	+	+ +	+	+	×	—	—	$+^{2}$	$+^{1,2}$	+
S hisingen — 48 a 1 5 7	+	+	-	—	+	+	+	+	—	-	—	—	—	×	+
S wassenaar — 50 g (p) —	+	—		$+^{3}$	+	+	-	+	+	×	—		+°	—	—
S bonaire — 50 z_4 z_{2} —	+		—	+	+	+	—	+	+	—	—		$+^{2}$	—	—
S antsalova — 51 z 1 5	+	+	+	+	+	+	+ +	+	—	+	×	—	+	+	—
S sainte marie — 52 g t —	+	+	—	+	+	+	+ +	+	—	—	—	—	$+^{2}$	+	—
*Salmonella — 52 — 1 5 7	+	+	—	+	+	+	+ +	+	+	×	—	—	$+^{2}$	+	+
S uccle — 54 g s t —	+	+	—	+	+	+	—	—	—	+	+	—	+	+	—
*S tranoroa — 55 k z_{39}	+	+	—	+	+	+	+ +	+	+	—	—	—	$+^{2}$	+	+
*S artis — 56 b —	+	+	—	+	+	+	+ +	+	+	—	—	—	$+^{2}$	+	+
*S locarno — 57 z a z_{42}	+	+	+	+	+	+	+ +	+	+	$+^{2}$	—	—	$+^{2}$	+	+
*S basel — 58 l z_{13} z_{28} 1 5	+	+	—	+	+	+	+ +	+	+	×	—	—	$+^{2}$	+	+

Key Ara — arabinose Dul — dulcitol Ino — inositol Rha — rhamnose Tre — trehalose, Xyl — xylose Gly — Stern's glycerol fuchsin broth Gel — gelatin d — d tartrate, l — l tartrate, i — i tartrate Cit — sodium citrate Muc — mucate Mal — sodium malonate — Arabinose to xyloses + — positive after 1 day — — negative after 30 days, × — late and irregularly positive or negative d — different biochemical types — Stern's glycerol fuchsin broth + + lilac after 1–2 days, — negative after 8 days — Gelatin + — slowly positive in ferrous chloride gelatin and rapidly positive in the Kohn test modified by Lautrop — Organic acids + — positive after 1 day, $+^{2,3}$ — positive after 2 3 days — — negative after 14 days * — sub-genus II

S hisingen fermented sorbitol after 2 days

Since some of the above mentioned *species* are strongly deviating from the typical, biochemical behaviour, they are summarized in Table 1

List of Salmonella Species Recognized in 1961

Salmonella adamstown = 28 k 1,6
Salmonella agona = 4,12 f,g,s —
Salmonella alsterdorf Rohde & Bischoff = 1,40 g m t —
Z Hyg in press
Salmonella antsalova = 51 z 1,5
Salmonella artis = 56 b —
Salmonella bahrenfeld Rohde & Bischoff = 6,14,24 e,h 1,5
Z Hyg in press
Salmonella bamboye = (9),46 b l,w
Salmonella basel Rohde, Fey & Steck = 58 l,z_{13} z_{28} 1,5
Zbl Bakter I Orig in press
Salmonella bedford = 1,3,19 l,z_{13},z_{28} e,n,z_{15}
Salmonella benfica = 3,10 b e,n,x
Salmonella bergedorf Rohde & Bischoff = (9),46 e h 1,2
Z Hyg in press
Salmonella bilthoven = 47 a —
Salmonella bonair = 50 z_4,z_{30} —
Salmonella ceres = 28 z z_{39}
Salmonella chincol = 6 8 g,m,s e,n,x
Salmonella chudleigh = 3,10 e n,x 1,7
Salmonella egusi = 41 d —
Salmonella egusitoo = 1,42 b z
Salmonella elsiesrivier = 16 z_{40} 1,6
Salmonella fandran = 1,40 z_{35} e,n,x,z_{15}
Salmonella finchley = 3,10 z e n,x
Salmonella fischerkietz Rohde Schneider & Goldberg =
1,6 14,25 y e n,x
Monatshefte fur Veterinärmedizin in press
Salmonella fischerstrasse = 44 d e,n z_{15}
Salmonella foulpointe = 38 g t —
Salmonella hisingen Backelin, Heilborn & Rutquist = 48 a 1,5,7
Salmonella houten = 43 z_4 z_{23} —
Salmonella hueningen Rohde, Fey & Steck = 9 12 z z_{39}
Zbl Bakter I Orig in press
Salmonella huila = 11 l,z_{28} e n,x
Salmonella islington = 3,10 g t —
Salmonella katesgrove = 1,13,23 m,t 1 5
Salmonella kiwu van Oye, van Ros & Herman = 6 7 d 1,6
Annal Inst Pasteur *100*, 812, 1961

Salmonella linton = 13,23 r e,n,z_{15}
Salmonella locarno Kauffmann, Fey & Steck = 57 z_{29} z_{42}
Acta path et microbiol scandinav *53*. 423, 1961
Salmonella lundby Backelin, Heilborn & Rutquist = (9),46 b e,n,x
Salmonella makumira Rohde & Muller = 4,12 e,n,x 1,7
Z Hyg in press
Salmonella mampong = 13,22 z_{35} 1,6
Salmonella mim = 13,22 a 1,6
Salmonella mocamedes = 28 d e,n,x
Salmonella neumuenster = 1,4,12,27 k 1,6
Salmonella norton = 6,7 i l,w
Salmonella nowawes = 40 z z_6
Salmonella ohlstedt Rohde, Adam & Bischoff = 3,10 y e,n,x
Z Hyg in press
Salmonella portbech = 42 l,v e,n,x,z_{15}
Salmonella sainte-marie = 52 g,t —
Salmonella soahamina = 6,14,24 z e,n,x
Salmonella soesterberg = 21 z_4,z_{23} —
Salmonella suarez = 1 40 c e,n,x,z_{15}
Salmonella tafo = 1,4,12,27 z_{35} 1,7
Salmonella techiman = 28 c z_6
Salmonella tournai van Oye, Le Minor & Moinet = 3,15 y z_6
Annal Inst Pasteur *101*, 135, 1961
Salmonella tranoroa Le Minor, Le Noc & Drean = 55 k z_{39}
Annal Inst Pasteur *101*, 133, 1961
Salmonella tuindorp = 43 z_4,z_{32} —
Salmonella uccle Kauffmann, Thomas & van Oye = 54 g,s,t —.
Acta path et microbiol scandinav *53*, 201, 1961
Salmonella vietnam = 41 b —
Salmonella vleuten = 44 f,g —
Salmonella volta = 11 r l,z_{13},z_{28}
Salmonella wa = 16 b 1,5
Salmonella wandsbek Rohde & Bischoff = 21 z_{10} z_6
Z Hyg in press
Salmonella wassenaar = 50 g,(p) —
Salmonella wernigerode = (9),46 f,g —
Salmonella wippra = 6,8 z_{10} z_6
Salmonella zehlendorf = 30 a 1,5
Salmonella zeist = 18 z_{10} z_6
Salmonella = 4,5,12 g,(p) 1,7
Salmonella = 4,12 z_{36} —
Salmonella = 6,8 c 1,2
Salmonella = (9),46 g,s,t —
Salmonella = 13,23 i e,n,z_{15}
Salmonella = 52 — 1,5,7

Addendum to Supplement IV
(*Kauffmann* 5)

Salmonella canada = 4,12 b 1,6
Salmonella gilbert = 6,7 z_{39} 1,7
Salmonella sullivan = 6,7 z_{42} 1,7
Salmonella bahati = 13,22 b e,n,z_{15}
Salmonella woodstock = 16 z_{42} 1,(5),7
Salmonella kuessel = 28 i e,n,z_{15}
Salmonella luckenwalde = 28 z_{10} e n,z_{15}
Salmonella westphalia = 35 z_4 z_{24} —
Salmonella lichtenberg = 41 z_{10} z_6

Since a diphasic culture of *S adabraka* was isolated, the formula of this *species* is given as 3,10 z_4,z_{23} 1,7

SUMMARY

A supplement to the *Kauffmann White Schema* is given, containing 69 new *Salmonella species* recognized during the year 1961

REFERENCES

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A ... the genus Salmonella Acta

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Kauffmann F (5) Supplement to the Kauffmann White Scheme (IV) Acta path et microbiol scandinav *52* 221 1961

FROM KAPTEIN W. WILHELMSEN OG FRUES BAKTERIOLOGISKE INSTITUTT, OSLO UNIVERSITY AND RIKSHOSPITALET, OSLO

SOME PASTEURELLA STRAINS FROM THE HUMAN RESPIRATORY TRACT

A Correction and Supplement

By

S. D. HENRIKSEN

Received 24.i.62

In a previous study (1) the only strain of Pasteurella pneumotropica which was available (Strain 12555 from the American Type Culture Collection) was f...

fermentation in the original description of the species (2), it was not realized that our result was at variance with the expected behaviour of this species.

Later my attention was called to the fact that elsewhere this strain and other strains identified as P. pneumotropica had been found invariably mannitol negative, and that, consequently, two of our strains, 953/60 and 4225/60, which we had considered as a special biotype of P. multocida, actually might be P. pneumotropica (3). This information made a renewed study of these strains necessary.

MATERIAL

In addition to the 3 strains mentioned above a strain of P. pneumotropica 6184 received from Miss *Elizabeth O. King*, Atlanta, was included in the study.

METHODS

Several different fermentation media were tried. Good growth ...

... methods were as before.

RESULTS

Morphologically the strains were similar, although 12555 tended to produce longer rods than the others. Agar cultures of strains 12555 and

I am indebted to Miss *Elizabeth O. King*, Atlanta, for friendly advice and for pasteurella strains.

Addendum to Supplement IV
(Kauffmann 5)

Salmonella canada = 4,12 b 1,6
Salmonella gilbert = 6,7 z_{39} 1,7
Salmonella sullivan = 6,7 z_{42} 1,7
Salmonella bahati = 13,22 b e,n,z_{15}
Salmonella woodstock = 16 z_{12} 1,(5),7
Salmonella kuessel = 28 i e,n,z_{15}
Salmonella luckenwalde = 28 z_{10} e n,z_{15}
Salmonella westphalia = 35 z_4,z_{24} —
Salmonella lichtenberg = 41 z_{10} z_6

Since a diphasic culture of *S adabraka* was isolated, the formula of this *species* is given as 3,10 z_4,z_{23} 1,7

SUMMARY

A supplement to the *Kauffmann White Schema* is given containing 69 new *Salmonella species* recognized during the year 1961

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[illegible] sions of the genus Salmonella Acta
0
Kauffmann F (4) The species [illegible] he family Enterobacteriaceae Internat Bull Bacter Nomen & Taxon *11* 5 1961
Kauffmann F (5) Supplement to the Kauffmann White Scheme (IV) Acta path et microbiol scandinav 52 221 1961

FROM THE DEPARTMENT OF VIRUS RESEARCH KAROLINSKA INSTITUTET, STOCKHOLM AND THE DEPARTMENT OF CLINICAL CHEMISTRY AND THE BLOOD TRANSFUSION SERVICE UNIVERSITY HOSPITAL UPPSALA SWEDEN

THE REACTIVITY OF BLOOD GROUP A ISOANTIBODIES FRACTIONATED BY DEAE–CHROMATOGRAPHY AND GEL FILTRATION IN THE "MIXED AGGLUTINATION" AND "SEROLOGICAL ADHESION" TESTS

By

CLAES F HOGMAN and JOHAN KILLANDER

Received 6 ii 62

A reaction between human blood group A or B antibody and the homologous antigens present on the surface of tissue cells can be detected by several serological methods, *e g* mixed agglutination (abbreviated below as MA) (4 11) and serological adhesion (abbreviated below as SA) (14) In both these methods blood group antibodies are fixed to antigens present on the surface of the tissue cells and red cells are used to indicate the antigen antibody reaction In MA the red cell surface antigen (ABO) combines directly with the antibody, in SA human red cells, irrespective of their ABO blood group adhere to the tissue cells in the presence of certain complement components (6)

Not all sera containing, *e g*, group A antibodies give a MA reaction with group A tissue cells (10,12) This may suggest that an anti-A which can combine with the group A antigen of human red cells does not always react with group A antigen of other human tissues Evidence has been presented (10, 13, 24) that the group A antigen of certain tissue cells is identical with group A^P antigen which represents only part of the whole group A antigenic structure of the human erythrocytes However, another explanation for negative MA reactions may also be conceivable fixation of antibody may have occurred without being detectable by the method used Therefore it seemed to be of interest to investigate if the SA method would detect any group A—anti A reactions not demonstrable with MA

In addition to the serological heterogeneity of group ABO [illegible]
bodies [illegible]
al (9) [illegible]
with se[illegible] [illegible] can occur

This study was aided by grants from the *Swedish Cancer Society* and the *Medical Faculty of the University of Uppsala*

For excellent technical assistance the authors are indebted to Miss *Siv Andersson* Mrs *Gerd Olofsson* and Miss *Britt Westerdahl*

6184 showed a more distinct yellowish pigmentation than the two others, but otherwise appearance and odour of the cultures were similar The strains gave a positive oxidase reaction within 10 seconds after flooding agar cultures with a c 0 2 per cent solution of tetramethyl p phenylenediamine All strains fermented glucose, maltose, fructose and saccharose, but failed to attack mannitol, dulcitol, sorbitol, rhamnose and salicin Slight acidity was produced from glycerol Strains 12555 and 6184 fermented lactose, xylose and arabinose in both media Strains 953/60 and 4225/60 caused a slight acid production, detectable only in the peptone-bromthymol blue medium from xylose, and a very questionable reaction with arabinose in the same medium

All strains reduced nitrates, split urea and produced indol and small quantities of H_2S, and gave negative methyl red and Voges-Proskauer reactions

COMMENT

The previously reported mannitol fermentation of strain 12555 seems to have been a technical error Also, when more adequate basic media are used, the strain ferments xylose and arabinose The differences between our two strains 953/60 and 4225/60 and the two strains of P pneumotropica can not be considered as sufficient to justify separation of these strains in different species Thus fermentation both of lactose and of arabinose has been shown to give variable results in this species (2, 3) It appears, therefore, that our two strains from the human respiratory tract should be classified as Pasteurella pneumotropica

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Gel filtration was performed on cross linked dextran (Sephadex) with "water regain" (Wr) of 20 g (G 200) and 15 g of water per g gel substance as described by *Flodin* (7) and *Killander & Flodin* (16 17)[2] In experiments No 10-12 6 to 9 ml of serum or electrophoretically isolated beta-gamma globulins (starch block electrophoresis) from corresponding amounts of sera was separed on colmns with a diameter of 3 0–3 8 cm and a length of 43–46 cm The buffer used was 0 1 M trishydroxymethyl aminomethane (TRIS), pH 8 0, containing 0 2 M NaCl In experiments No 13–17 7 ml of serum or electrophoretically fractionated beta gamma globulins from sera was fractionated on Sephadex G 200 columns with a diameter of 3–4 cm and a length of 38–46 cm in 0 1 M TRIS buffer, pH 8 0, containing 1 M NaCl

The protein content of the serum appeared in three main peaks (cf Fig 1) The first contained mainly macroglobulins (19 S), the second mainly 7 S globulins and the last mainly albumin Some overlapping ocurred between the 19 S and 7 S

Immunoelectrophoresis was performed according to *Scheidegger* (22)

described by Kunkel (15) in a Spinco Model L ultracentrifuge

Serological Methods

Human kidney cell cultures from group A or AB fetuses were tested by the MA and SA techniques described in detail previously (11, 12, 14) The main steps in the reactions are presented here

Mixed agglutination (MA)

1 Sensitization of kidney cells with serial dilutions of serum fraction to be tested

2 Washing

3 Incubation with red cells of groups A_1 B and O (The group O cells were derived from the same donor as the one used in the SA test)

4 Reading under the microscope

Serological adhesion (SA)

1 Sensitization of kidney cells with serial dilutions of serum fraction to be tested

2 Washing

3 Incubation with fresh group AB serum diluted 1/20–1/30 (as a source of complement)

4 Washing

5 Incubation with group O red cells from one selected donor

6 Reading under the microscope

In order to ascertain that the SA activity was due to anti-A the fractions were tested with group B or O tissue when available, which served as a negative control In other experiments controls were obtained by absorption of the fractions with

testing serial two fold dilutions of the in 0 14 M NaCl and in the dextran-

[2] The gel filtration fractionation was performed by *Dr P Flodin, Research Laboratories AB Pharmacia Uppsala Sweden*

simultaneously in a serum By ion exchange chromatography (1, 2 5 19, 21, 23) it has been demonstrated that anti-A of the 7 S and the 19 S types can be separated from each other and that a serological hetero geneity exists in at least the 7 S antibody fractions (18)

In the present investigation human blood group A antibodies have been separated by means of anion exchange chromatography and by a recently described technique, gel filtration (7, 16) The 7 S and 19 S fractions have been tested by means of MA and SA technique on human foetal kidney cells A preliminary report of the results has been given by *Hogman et al* (15)

MATERIAL AND METHODS

Human serum was obtained from donors who had been immunized either by pregnancy or by injection of blood group A or B soluble substance (Knickerbocker Biosales New York) *Nine group O and four group B sera were used, two of each* being pools the others being derived from *single individuals*

Fractionation and Characterization of Antibody Globulins

Chromatographic separations were performed on columns packed with granules of cross linked dextran coupled with DEAE DEAE Sephadex A 50 Medium total anion exchange capacity 3 9 meq/g (8) (kindly supplied by AB Pharmacia Uppsala Sweden) *After washing as described by the manufacturer and equilibration* with the starting buffer (0 02 M phosphate pH 8 0) serum or electrophoretically prepared beta and gamma globulins[1] were placed on the top of the columns the size of which varied between 1 0–1 9 cm in diameter and 10–20 cm in length The amount of protein to be fractionated corresponded to 1 ml of serum for a column size of 1 cm × 10 cm Before application the sample was equilibrated with the starting buffer through dialysis or gel filtration on Sephadex G 25 with the exception of experiment No 9 in which the native serum was diluted only with one volume *of the buffer In this experiment and in others not referred to in the* present paper the gel filtering efect of the DEAE Sephadex substance was used besides the chromatographic effect Depending on this gel filtering effect such proteins of the serum as were unaffected by the DEAE groups appeared in the eluate *before the* electrolytes of the same serum were eluted Thus is was possible to separate the antibodies unaffected by DEAE in 0 02 M buffer without previous dialysis of the serum Antibodies which easily lose activity during dialysis and further procedures could be separated in this way more rapidly and with less or no loss of activity

The fraction eluted with the starting buffer was called fraction I Further elution was performed stepwise or with a continuously increasing gradient up to 0 3 M phosphate buffer pH 8 0 or 0 1 M phosphate pH 8 0 in 1 M NaCl The anti body containing fractions *eluting at higher molarity than 0 1 M phosphate* were pooled and were called fraction II Both fractions were concentrated by ultrafiltra tion and dialyzed against 0 14 M NaCl prior to the serological tests

The separation method with DEAE Sephadex chromatography was repeatedly tested using different sera containing anti A incomplete anti Rh or high titre cold *agglutinins* Re chromatography of several fractions was performed The fractions were analyzed immunoelectrophoresis and by zone ultracentrifugation in a sucrose density gradient Antibody active gamma globulin of the 19 S type was found to be eluted with *phosphate* buffer above 0 14 M *Gradient* elution experiments demonstrated that most of the gamma globulins of the 7 S type was eluted with 0 02 M phosphate buffer However a minor amount sometimes prossessing antibody activity in low titre *appeared at higher ionic strength Thus some group* A antibody of 7 S type was found to be eluted with 0 1 M phosphate buffer

[1] Zone electrophoresis on large preparative polyvinylchloride acetate (Pevikon) columns was kindly performed by Dr *J Porath Dept of Biochemistry University of Uppsala*

first peak was spun and only slightly above the middle of the tube when the second peak fraction was analyzed By immunoelectrophoresis and diffusion in-gel technique using anti-total-human serum and specific anti beta₂M serum the first fraction (A in Fig 1) was shown to contain beta₂M globulin (19 S gamma globulin) With specific anti-gamma (7 S) globulin serum a faint precipitation line was observed in the immunoelectropherogram at the site of the beta₂M line In diffusion in gel experiments using the anti gamma-globulin, serum fraction and pure 7 S gamma globulin gave each one precipitation line only which showed a reaction of partial identity This indicates that fraction A contained beta₂M globulin which cross-reacted with anti-gamma globulin serum and that 7 S gamma globulin was not present in detectable quantities By the same methods the fraction from the second part of the second peak (D in Fig 1) was shown to contain 7 S gamma globulin but no demonstrable beta₂M globulin No attempts to identify beta₂A globulin in the fractions were made

The Capacity of the Antibody Fractions to Cause MA and SA

The serological results obtained with the chromatographically separated fractions are summarized in Table 1 The SA technique was not established during the first part of the investigation In experiments 6–9 only the "7 S" fraction was tested The first fraction, containing antibodies of the 7 S type, caused MA in four out of nine experiments and in low titres only, SA was obtained in all of six experiments in titres up to 1/32 The second fraction, containing antibodies mainly of the 19 S type, caused MA in all of six experiments with titres up to 1/16 Similar results were reached by the SA method

TABLE 1

Titre of Antibody Causing MA and SA Reactions in Fractions Obtained by DEAE Sephadex Chromatography

Experiment no	Serum or serum fraction	Antibody tested	Titre of antibody: Fraction I (7 S type antibody) MA	SA	Fraction II (19 S type antibody) MA	SA
1	L M	anti A	1	–	16	–
2	K	anti B	0	–	8	–
3	K	anti A	0	–	1	–
		anti B	0	–	8	–
4	Ir *	anti A	0	16	4	2
5	Group B pool*	anti A	0	1	16	32
6	S F *	anti A	0	4	–	–
7	Gr §	anti A	2	16	–	–
8	T J §	anti A	< 8	32	–	–
9	Dan	anti A	4	> 16	–	–

* Electrophoretically prepared beta gamma globulin
§ Rechromatographied fractions

RESULTS

Fractionation Experiments

By *DEAE-Sephadex chromatography* two fractions were prepared Fraction 1, eluted with 0 02 molar phosphate buffer contained most of the antibodies of the "7 S" type Fraction 2, eluted with buffer of a molarity above 0 1 contained the "19 S" antibodies but may have contained some antibodies as well of the 7 S type in low titre

By *gel filtration* on cross-linked dextran antibodies of 7 S and 19 S types were separated according to their molecular sizes (Fig 1) The physicochemical type of the antibodies in the preparations was checked by density gradient ultracentrifugation, which revealed antibody activity only in the bottom of the centrifuge tube when fraction from the

Fig 1

7 ml of serum was separated on a 4 0×38 0 cm column packed with Sephadex G 200 (Mesh 200–270) in 0 1 M TRIS buffer containing 1 M NaCl, fraction volume 5 ml, flow rate 35 ml/hour The experiment was completed in 8 hours As indicated in the figure the fractions containing protein were pooled concentrated to 3 5 ml each dialyzed and subsequently tested by physicochemical immunochemical and serological methods

In the SA tests group O kidney cells were used as negative control

DISCUSSION

The most effective separation of the 7 S and 19 S antibody moieties was obtained by the gel filtration method no detectable amounts of 19 S gamma globulin were present in the 7 S preparations and no 7 S antibody activity in the 19 S preparations as judged from density gradient ultracentrifugation, immunoelectrophoretic and diffusion-in gel examinations In the DEAE chromatographic experiments the 7 S fractions were free of 19 S material but did not contain all 7 S antibodies The 19 S fractions may have been contaminated with small amounts of 7 antibodies, at least in some of the experiments However, when tested by MA and SA the corresponding gel filtration and chromatographic fractions gave the same reaction pattern which indicates that this contamination of the 19 S fractions did not markedly influence the results

A consistent finding in the serological tests was that the antibodies in the 7 S fractions did not cause MA at all or only in a very low titre That the fractions nevertheless contained antibody able to react with the kidney cells was demonstrated by the SA technique On the other hand the 19 S type of antibody gave positive reactions by both methods Thus, antibodies of different molecular sizes seemed to give different reaction patterns in the serological tests

The number of experiments and sera tested was too small to permit of any definite conclusions to be drawn concerning the possible presence of serologically heterogeneous antibodies of one and the same physico chemical type The fact that a negative reaction on tissue cells was obtained by MA as well as SA, although the fraction in question contained anti A, might indicate that this can be the case, however

SUMMARY

Human sera containing anti A have been fractionated by chromato graphy on DEAE coupled to cross-linked dextran (DEAE-Sephadex) and by gel filtration on crosslinked dextran (Sephadex, water regain 20 (G 200) and 15 g g)

Thus antibodies of the '7 S' and the "19 S" types could be separated and were tested with human foetal kidney cells by mixed agglutination and serological adhesion techniques The 7 S type of antibody did not cause mixed agglutination, or did so only in a low titre, but caused

19 S type of anti-

...ether or not a blood group A isoantibody causes mixed agglutination may in part be correlated with a physico-chemical property such as its molecular size

The tests with fractions obtained by gel filtration are demonstrated in Table 2 The antibodies of the 7 S type caused MA *in two out of* eight experiments and in both cases in a very low titre, only By the SA method positive reactions were obtained in six out of eight experiments with titres up to 1/32 In two experiments the reactions were negative by both techniques although group A antibodies were demonstrable by direct haemagglutination tests The titre in dextran serum solution was 1/160 in one of these experiments (No 15)

TABLE 2

Titre of anti-A Causing MA and SA Reactions in Fractions Obtained by Gel Filtration on Cross Linked Dextran (Sephadex)

Experiment no	Serum or serum fraction	Titre of antibody			
		7 S fraction		19 S fraction	
		MA	SA	MA	SA
10	Dan *	0	0	8	4
11	Dan	0	16	≥1	2
12	*Dan*	*0*	*32*	*≥1*	4
13	R S	0	≥1	0	0
14	Group O pool*	1	20	0	1
15	Nym	0	0	20–40	20–40
16	Group B pool	1	8	2	2
17	UBJ	0	20	20	20

* Electrophoretically prepared beta gamma globulin

The fractions containing antibodies of the 19 S type caused MA *in* six and SA in seven out of eight experiments, the titres being of the same order of magnitude by both methods

In figure 1 a representative experiment is demonstrated The 7 S fraction (fraction D) only showed SA activity, the 19 S fraction (fraction A) both *MA* and *SA* and *the intermediate fractions a continuous* decrease of the MA activity The reaction pattern of the MA reaction was similar to that of the "saline" haemagglutinin activity whereas the SA pattern resembled that of the "dextran-serum" haemagglutinin activity Whether the serological activity in this and in other experiments reflected a summation of the activities of two overlapping fractions or it was caused by a separate antibody fraction with physicochemical characteristics deviating from the 7 S and 19 S moieties remains uncertain

It may be reported finally that the haemagglutinating activity of the 7 S fractions always was lower in 0 14 M NaCl than in dextran-serum solution, the difference being up to seven titre steps The same was true for the 19 S fraction but the difference here was less pronounced (4 titre steps or less)

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"ASYMPTOMATIC" BACTERIURIA IN FEMALE PATIENTS IN THE MEDICAL WARD[1]

A Comparison between Mid stream Voided and Catheterized Urine specimen

By

SIMO VIRTANEN and ANTERO KASANEN

Pyelonephritis is a very common complaint Some 12–13 per cent (12, 18) of the female patients in medical wards are affected with it, and sometimes even 29 per cent (27) It is the most common kidney disease found at autopsy (24), its incidence in autopsy materials ranges from 2 8 per cent (25) to 9 per cent (17) and as high as 15–20 per cent (24) The incidence of pyelonephritis hos not declined since the introduction of antibiotics (17)

Active pyelonephritis, however, escapes clinical diagnosis regrettably often, even in 70–80 per cent of the cases (22, 28) Only in 16 7 per cent of Raaschou's autopsy material (31) was the correct diagnosis made The correct clinical diagnosis fails in cases with extensive renal changes as often as in those with slight renal lesions (21)

Obviously the reason is that pyelonephritis not always gives direct symptoms Fifty per cent of the patients in the series of *Jackson et al* (17) showed no acute symptoms at the time of diagnosis Fifty seven per cent of the pyelonephritis patients in the *series of Kasanen et al* (19) were subjectively symptom free In the series reported on by *Halonen & Halonen* (12), 64 6 per cent were found to have pyelonephritis free of subjective symptoms, it was more common among elderly than among young women Pyelonephritis may be symptomless for a long time until hypertension or renal insufficiency draws attention to the matter (8, 24, 34)

The objective diagnosis of pyelonephritis has been based primarily on study of the urinary sediment A drawback to this, however, is that urinary findings in pyelonephritis vary from time to time (5, 31) The incidence of pyuria, such as it is usually looked for, is not an essential feature of pyelonephritis (22) Absence of [illegible]

1 This study was supported by [illegible] and the Sigrid Juselius Foundation

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TABI I 1

(mjar son let een M istre 1 le l an l C th ter e l Ur ne Sp c mens f 165 Fe ales in the Me l cal Ward

Mi l tream i l pe men	Cath eriz l pec m n										
	0	1 10	10 10–	10 1 3	10 10	10^4 10^5	10^5–10^6	10^6–10^7	10^7 10^8	$>10^8$ rganis ns ml	Total
0	22										22
1 10	3										3
1 1	18										18
10 10^3	37										37
10^3–10^4	34		3								37
10 1 5	13		1		1						15
	127										
1 5–10	6					3					9
10^6–10^7	5		1								6
10^7 10^8	5		1						1		7
>10 kani n s/ml	6			1					1	3	11
	22										
T tal	149			1	1	3			2	3	165

existence of a urinary tract infection, nor is its presence evidence of a pyogenic infection (33) In fact, the examination of urinary sediment by the routine methods is of limited significance (14)

Thus the point of central importance in the diagnosis of pyelonephritis is the establishment of bacteriuria by bacteriological investigation Obtaining a reliable bacteriological specimen, however, is complicated by the fact that the lower urethal passage contains bacteria (3) *Hirshfeld et al* (13) found bacteria in the urethras of about 50 per cent of 114 pregnant women Just how far up in the urethral passage bacteria ordinarily lie is not definitely known, but in a study of normal men bacteria were found at distances of 7 to 14 cm from the meatus (6) This area cannot be made reliably sterile for the collection of a specimen (3) Therefore, a positive culture often will be due to contamination (11) In addition, the risk is involved of bacteria being carried to the upper urinary tract by the catheter The iatrogenic infection percentage on catheterization, according to *Kass* (21, 23) is 2–4, in *Marple's* series 8 7 (29), and in the series reported by *Davis et al* up to 29 per cent (4) The risk is particularly great for diabetics and patients with urinary tract anomalies or obstructions The long term risk of complications from the catheterization of a diabetic patient approximates that of a thyroidectomy (32) Definite hospital infections (7, 26, 39, 40) in which the patient has been infected by a drug-resistant bacterial strain (7, 26) have also been reported

True bacteriuria, however, can be distinguished from contamination in specimens by employing quantitative culture methods in which the total of bacteria per ml of urine is counted The counting may be done either from a catheterized specimen, or from a mid-stream voided specimen which obviates the risks of catheterization

For a catheterized specimen, a count of over 10,000 bacteria per millilitre was taken as true *bacteriuria (1, 4, 30) For the mid stream* voided specimen, 50,000–100,000 bact/ml (30, 36) and over 100,000 bact/ml (16, 22) have been considered to indicate true bacteriuria

SERIES AND METHOD

The series comprised 165 female patients of the Medical Clinic of Turku University Patients only were included whose medical histories indicated no urinary tract infections or other urinary tract complaints The mean age of the patients was 47 8 ± 1 2 years

Specimens were taken on admission The external genitals and the perineum of the patients was first washed with soap and water as usual upon which the area of the urethral meatus was cleansed with boiled water When the patient passed urine a mid stream specimen was taken in a sterile container of stainless steel from which it was poured into a sterile test tube After the specimen had been obtained the patient was asked to stop and after cleansing the external genitals she was catheterized with a metal catheter The catheters were sterilized in hot air in an oven at 170° C for one hour and the sterility was checked from time to time

Both specimens were sent immediately to the Department of Medical Microbiology University of Turku where they were kept in a refrigerator until tested The study and culture of urinary sediment were done on the same day not later than 2–3

cases the voided specimens showed > 100,000 bact./ml. In 22 cases, 14.8 (± 2.9) per cent, over 100,000 bact./ml were present in the voided specimen although the catheterized speciment was completely negative. If the limit of significant bacteriuria is set at > 10,000 bact./ml in catheterized urine, a significant bacteriuria finding contradictory to the one in the catheterized specimen was found in a total of 25 voided specimens: 16 (± 2.9) per cent.

The bacterial strains listed in Table 2 were isolated in 135 cases for which the finding in the voided specimens could not be considered as significant bacteriuria. As can be seen, these contaminants included plenty of potential pathogens.

All the 8 cases of true bacteriuria were provoked by a single strain, seven by E. coli and one by Enterococcus.

DISCUSSION

Kass found asymptomatic bacteriuria in 6 per cent of the women visiting a medical outpatient department (20) and 6 per cent of pregnant women making their first prenatal visit (23). *Marple* (29) found bacteriuria in 23 per cent of an unselected group of hospitalized women. *Merrit & Sanford* (30) found bacteriuria in 14 per cent of a consecutive series of hospitalized women, and *Boshell Sanford* (1) in 15 per cent. The proportion for the present series, 5 per cent of asymptomatic bacteriuria, is very close to *Kass*'s figure. The percentages in different series are not directly comparable since the type and composition of the series may differ greatly. In addition, the present series, like the one reported by *Kass* was selected and comprised only asymptomatic patients, while the other series mentioned above were unselected.

The confidence limit of a single bacterial count, according to *Kass* (23) was about 80 per cent. In the present material, the confidence limit of a single bacterial count was 84 per cent. Theoretically, therefore 97 per cent confidence should be achievable by testing two sterile voided mid stream specimens, a confidence very close to that of a single catheterized specimen which, according to *Kass* (23), is 95 per cent. Whether this holds good in practice has not been demonstrated so far.

Of the 8 cases in which significant bacteriuria was diagnosed from the catheterized specimen, 3 must be considered subjectively asymptomatic pyelonephritis. The diagnosis in these cases was based on a simultaneously verified high incidence of leucocytes in the urinary sediment.

Persistent bacteriuria is an early symptom of pyelonephritic patients. It is not synonymous with pyelonephritis, but a certain percentage (not known to date) of these patients develop pyelonephritis (15). But manifest pyelonephritis need not develop in all of the cases in which permanent bacteriuria is found (37). Bacteriuria is also seen in cases of treatment failure and relapse (9).

A considerable drawback of the voided specimens was that nearly

hours after taking the specimen The sediment was examined in a cover glass preparation it was Gram stained and cultured on brom cresol purple lactose agar plate 5 per cent sheep s blood agar plate and a sheep s blood agar plate containing 0 08 per cent thallium acetate (for streptococci) The bacterial count was taken by the pour plate method mixing urine in nutrient agar The urine dilutions on the plates were 10^{-1} 10^{-2} — — — 10^{-5} The plates were incubated for 24 hours at +37° C In some cases where the colonies on the pour plates were small the count was rechecked after an incubation of 48 hours The following biochemical reactions were done to identify the gram negative rods mannitol and glucose fermentation and gas formation lactose fermentation indole production hydrogen sulphide production and gelatine liquefaction urea decomposition citrate utilization methyl red reaction and the KCN test The motility was followed in a Graigie tube If required a litmus milk test was made growth at 44° C tested and the pigment formation observed on an ordinary agar plate

For staphylococci mannitol fermentation and coagulase production were studied (35) for streptococci growth in 6 5 per cent sodium chloride broth

Gram positive rods were tested for survival for 24 hours in acetic acid broth of pH 3 for the presence of metachromatic granules by Bie s stain fermentation of glucose sucrose dextrin and starch and growth at room temperature

All of the bacterial strains isolated from the catheterized specimens were tested by the disc method for sensitivity to the following drugs penicillin streptomycine chloramphenicol tetracycline sulfa nitrofurantoin and methenamine mandelate

Urinary sediment Gram s stain and cultures were studied and recorded blind without the investigator (S V) knowing the results of the previous tests

TABLE 2

Bacterial Strains Isolated from Mid Stream Voided Urine Specimens in 135 Cases of Non Significant Bacteriuria

Strain	Alone	With one other strain	With two other strains	Total
E coli	32	26	3	61
Klebsiella	4	1		5
Citrobacter	2	6		8
Achromobacter	2	2		4
Proteus	2	1	2	5
Diphtheroid	1	2		3
Lactobacilli	4	1		5
Staphylococcus albus	24	18	1	43
Staphylococcus aureus	2	2		4
Sarcina		1		1
Enterococci	8	15	2	25
Streptococcus α hemolyticus	6	12	1	19
Candida		3		3
Total (cases)	87 +	90/2=45 +	9/3=3 =135	186

RESULTS

The comparison of the catheterized and voided urine is given in Table 1 Only 22 voided specimens (13 3 per cent) but 149 catheterized specimens (90 5 per cent) gave a completely negative results The limits of significant bacteriuria are shown in the table by a blank > 10 000 bact./ml for catheterized specimens and > 100 000 bact./ml for voided specimens True bacteriuria (> 10 000 bact./ml in catheterized specimen) was found in 8 patients 4 9 (± 1 7) per cent In all of these

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40

35 per cent (Table 2) of the cultures were mixed ones Mixed cultures in catheterized specimens usually suggest an obstructive cause or complicating anomaly in the urinary tract (2, 10)

Among the disadvantages of a colony count is that enterococci and staphylococci may well be associated with low colony counts (38) According to *Jackson*, 5 per cent with low counts actually have chronic pyelonephritis (16)

SUMMARY

The mid-stream voided and catheterized urine specimens of 165 female patients in the medical ward were compared The confidence limit of a single mid-stream voided specimen was 84 per cent Asymptomatic bacteriuria was verified in 5 per cent of the patients

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number of patients with chronic active infection have acute exacerbations during pregnancy or the postpartum period (14) Acute pyelonephritis occurs in about 2 per cent of pregnant women, mostly during the latter half of pregnancy (1) The incidence of urinary tract infections during pregnancy, according to different authors, ranges from 0 5 to 16 per cent (3)

In a series of 615 parturients, *Jaämeri* (8) obtained a positive culture in 13 7 per cent of catheterized urine specimens Three quarters of these had no subjective symptoms

Pyelonephritis frequently masquerades as toxaemia of pregnancy Of Finnerty s series of 1,130 patients with suspected toxaemia, 6 5 per cent had pyelonephritis None of these patients complained of genitourinary symptoms (4) Renal infections were present in about 20 per cent of cases of toxaemia of pregnancy (14)

SERIES AND METHOD

The present series comprised 419 parturients 375 from Turku Municipal Maternity Hospital and 44 from Turku University Women s Clinic They had consulted the Maternity Welfare Centre in the normal way during pregnancy The mean age of the parturients was 25 4 ± 0 4 years 49 5 per cent were primiparas and 50 5 per cent multiparas

Group I 215 parturients comprised consecutive patients whose medical history contained no indications of urinary tract infection Group II 204 parturients were consecutive unselected admissions The mean age was the same for both groups Group I had 51 per cent and Group II 47 5 per

After the patients
water and the pubic
cent Bradosol (Ciba)
catheter

The urine sugar and albumen and haemoglobin of all of the parturients were tested routinely and their blood pressure was measured

The treatment of the specimens study of urinary deposit bacterial identification and count were performed as described earlier (24)

TABLE 1

The Results of Quantitative Urine Cultures of 419 Pregnant Women at Term

Organisms ml		0 10	10 10^2	10^2 10^3	10^3 10^4	10^4 10^5	10^5 10^6	10^6 10^7	10 10^8	$>10^8$	Significant bacteriuria per cent	Total
Group I (selected)	195	–	2	4	–	1	1	1	1	10	6 5	215
Group II (unselected)	170	3	16	4	3	5	1	–	–	2	3 9	204
	365	3	18	8	3	6	2	1	1	12	5.3	419

RESULTS

The results of the quantitative urine cultures are shown in Table 1 The limit for significant bacteriuria, > 10,000 organisms ml in a cathe-

FROM THE DEPARTMENT OF MEDICAL MICROBIOLOGY UNIVERSITY OF TURKU FINLAND (HEAD PROFESSOR EERO MUSTAKALLIO M D) THE WOMEN'S CLINIC UNIVERSITY OF TURKU (HEAD PROFESSOR SAKARI PARVIAINEN M D) AND THE MUNICIPAL MATERNITY HOSPITAL OF TURKU (HEAD ROBERT HASSELBLATT M D)

"ASYMPTOMATIC" BACTERIURIA IN PREGNANT WOMEN AT TERM[1]

By

SIMO VIRTANEN

Received 16162

Asymptomatic bacteriuria is a common infection which often precedes and predisposes to the development of pyelonephritis (12) Forty per cent of untreated pregnant women with bacteriuria developed pyelonephritis of pregnancy while none of those who were treated and no women without bacteriuria at the time of initial screening developed pyelonephritis of pregnancy (13) Pyelonephritis of pregnancy was preventable and bacteriuria had a predictive value in delineating the group of patients from which some will develop pyelonephritis (19) Forty six per cent of *Mulla* s (18) untreated bacteriuria patients developed clinical evidence of active infection only 6 per cent of the treated The treatment was of short duration one week only

The incidence of premature infants was over twice and that of neonatal deaths 5 times higher among the untreated women with bacteriuria than among the treated and the non bacteriuric controls (12)

Bacteriuria set in before the anatomical changes proper of pregnancy had developed for most if not all of the cases of bacteriuria were diagnosed before the second month of pregnancy (12) About 6 per cent of 2 000 pregnant women making their first prenatal visit were found to have asymptomatic bacteriuria (13) Bacteriuria was more common in multiparas than in primiparas rising to about 9 per cent in grand multiparas (11)

Urinary tract infection is often latent and insidious in its course (20) In over 50 per cent of the women the pyelonephritis was subjectively symptom free (7 9) During pregnancy it could simulate threatened abortion or premature delivery (17) The transient acute phase is often succeeded by a phase in which active infection persists and a chronic infection supervenes This phase may be latent or asymptomatic A

1 Aided by grants from the *Rockefeller Foundation* and the *State Commission for Medical Science*

number of patients with chronic active infection have acute exacerbations during pregnancy or the postpartum period (14) Acute pyelonephritis occurs in about 2 per cent of pregnant women, mostly during the latter half of pregnancy (1) The incidence of urinary tract infections during pregnancy, according to different authors, ranges from 0 5 to 16 per cent (3)

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SERIFS AND METHOD

The present series comprised 419 parturients 375 from Turku *Municipal Maternity* Hospital and 44 from Turku University Women s *Clinic They had* consulted the Maternity Welfare Centre in the normal way during *pregnancy The mean* age of the parturients was 25 4 ± 0 4 years 49 5 per cent were primiparas and 50 5 per cent multiparas

Group I 215 parturients comprised *consecutive patients* whose medical history contained no indications of urinary tract *infection Group II* 204 parturients were consecutive unselected admissions The mean age was the same for b th groups

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Group I (selected)	195	–	2	4	–	1	1	1	1	10	6.5	215
Group II (unselected)	170	3	16	4	3	5	1	–		2	3 9	204
	365	3	18	8	3	6	2	1	1	12	5.3	419

RESULTS

The results of the quantitative urine cultures are shown in Table 1 The limit for significant bacteriuria, > 10,000 organisms/ml in a cathe-

terized sample, is indicated by a wider vertical space in the table In group I, patients without a medical history of urinary tract infection the incidence of asymptomatic bacteriuria was 6 5 (± 1 7) per cent in group II, non selected patients 3 9 (± 1 4) per cent The difference is not statistically significant

TABLE 2

The Strains Isolated from 22 Cases of True Bacteriuria (> 10 000 organisms ml)

Strain	Alone	+ one other
E coli	15	2
Citrobacter	1	
Klebsiella	1	
Staphylococcus albus	3	1
Candida	–	1
	20	4/2 = 2

TABLE 3

The Strains Isolated from 32 Cases of Non Significant Bacteriuria (< 10 000 Organisms ml)

Strain	Alone	+ one other	+ two other
E coli	3	1	2
Klebsiella	1		
Proteus			2
Achromobacter	1		
Lactobacilli		1	
Staphylococcus albus	12		
Streptococcus α hemol	2		2
Candida	8	2	–
	28	4/2 = 2	6/3 = 2

TABLE 4

Pyuria Proteinuria and Blood Pressure in 22 Females with True Bacteriuria

Leucocytes in urinary deposit		Proteinuria		Blood pressure	
+(>al p f)	–	+	–	elevated (≥ 140/90)	normal
2	20	2 (transient)	20	2 (transient)	20

The incidence of significant asymptomatic bacteriuria in the total series was 5 3 (± 1 1) per cent For primiparas is was 5 8 (± 1 6) per cent and for multiparas 4 7 (± 1 5) per cent Among grand multiparas (quadriparas or more) the incidence of bacteriuria was 7 5 (± 4 2) per

cent These differences are not statistically significant Completely negative cultures were obtained from 87 1 per cent of specimens Non-significant bacteriuria was present in 7 6 per cent

The strains isolated from the true bacteriuria cases are listed in Table 2 E coli was the predominant organism The bacterial strains isolated from the cases of non significant bacteriuria are given in Table 3 As can be seen, the majority of the strains were micro organisms normally found on the mucous membranes of the genital tract

The principal clinical findings in the 22 patients with "true' bacteriuria are shown in Table 4 From the tests performed only 2 cases can be considered manifest pyelonephritis Of these 22 patients, 12 were primiparas and 10 multiparas

All of the children born to women with ' true ' bacteriuria were healthy and with one exception, born at term In the one exception delivery occurred a fortnight before the calculated time, and the child weighed 2 450 g

DISCUSSION

The incidence of eclampsia in all deliveries in the Province of Turku-Pori Finland, is 0 08 per cent (19) The records of Turku Municipal Maternity Hospital for 1955, 1957, and 1958 contain 7 cases of eclampsia per 4 721 deliveries i e 0 15 per cent Hence asymptomatic bacteriuria is about 35 times more common among parturients than eclampsia

Considering that pyelonephritis has a great tendency to recidivate during subsequent pregnansies (23) and that pyelonephritis, with few or no symptoms may become chronic and end in uremia and death (2 21) the early diagnosis of pyelonephritis is important For acute

per cent (6) According to *Colby* (2) the recovery percentage in chronic, complicated pyelonephritis was 27 7 in uncomplicated cases 14 5 per cent Since even in the best of cases the treatment of pyelonephritis is unsatisfactory it is of the utmost importance to diagnose and treat early a group showing a high incidence of pyelonephritis, *i e* the patients with asymptomatic bacteriuria

It has been found repeatedly that E coli and related enterobacteria are the most important causative organisms in pyelonephritis (2, 15) The same organisms predominate also in bacteriuria (Table 2)

The incidence of asymptomatic bacteriuria in the present series, 5 3 per cent concurs well with observations by *Kass* (13) in Boston, about 6 per cent According to *Kass* (11), bacteriuria is more frequent among multiparas than among primiparas In the present series no statistically significant correlation was obtained between bacteriuria and parity *Virtanen & Kasanen* (24) found asymptomatic bacteriuria in 5 per cent of the female patients in the medical ward The mean age of their

terized sample, is indicated by a wider vertical space in the table. In group I, patients without a medical history of urinary tract infection the incidence of asymptomatic bacteriuria was 6.5 (± 1.7) per cent; in group II, non-selected patients, 3.9 (± 1.4) per cent. The difference is not statistically significant.

TABLE 2

The Strains Isolated from 22 Cases of True Bacteriuria (> 10 000 organisms/ml)

Strain	Alone	+ one other
E. coli	15	2
Citrobacter	1	–
Klebsiella	1	
Staphylococcus albus	3	1
Candida	–	1
	20	4/2 = 2

TABLE 3

The Strains Isolated from 32 Cases of Non Significant Bacteriuria (< 10 000 Organisms/ml)

Strain	Alone	+ one other	+ two other
E. coli	3	1	2
Klebsiella	1	–	
Proteus			2
Achromobacter	1	–	
Lactobacilli		1	
Staphylococcus albus	12		
Streptococcus α hemol.	2		2
Candida	8	2	–
	28	4/2 = 2	6/3 = 2

TABLE 4

Pyuria, Proteinuria and Blood Pressure in 22 Females with True Bacteriuria

Leucocytes in urinary deposit		Proteinuria		Blood pressure	
+(>5 h.p.f.)	−	+	−	elevated (> 140/90)	normal
2	20	2 (transient)	20	2 (transient)	20

The incidence of significant asymptomatic bacteriuria in the total series was 5.3 (± 1.1) per cent. For primiparas it was 5.9 (± 1.6) per cent and for multiparas 4.7 (± 1.5) per cent. Among grand multiparas (quadriparas or more) the incidence of bacteriuria was 7.5 (± 4.2) per

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series was 47 8 ± 1 2 years In the present series of parturients with a mean age of 25 4 ± 0 4 years the incidence of asymptomatic bacteriuria was 5 3 per cent Evidently therefore a women s age between 25-48 years does not affect the incidence of asymptomatic bacteriuria

According to *Marple* (16) the past history of the urinary tract is not of great value because there is no striking difference in the histories of those with positive and those with negative cultures In the present series the past history was of no value for the diagnosis in bacteriuria patients The bacteriuria therefore was truly asymptomatic

As can be seen from Table 4 the examination for pyuria by routine methods and the tests for proteinuria are hardly of significance for diagnosing asymptomatic bacteriuria

All these parturients were followed during pregnancy at the Maternity Welfare Centre in the usual way It is obvious that with the methods of examination routinely used at present by maternity welfare centres (and many maternity hospitals) in Finland asymptomatic bacteriuria passes almost completely undetected

SUMMARY

419 parturients admitted to maternity hospital were examined for bacteriuria by quantitative culture of a catheterized urine specimen The culture result was completely negative in 87 1 per cent of the cases Significant bacteriuria (> 10 000 organisms/ml) was present in 5 3 ± 1 1 per cent Escherichia coli was the most common micro organism found in 77 5 per cent Non significant bacteriuria (< 10 000 organisms/ml) was found in 7 6 per cent

The importance of the early diagnosis of bacteriuria is discussed The conclusion reached was that asymptomatic bacteruria cannot be diagnosed by the standard methods of examination now used by maternity welfare centres in Finland

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TABLE 1

Comparison between Mid Stream Voided and Catheterized Urine Specimens of 215 Patients

Midstream Voided Specimen	Catheterized Specimen: 0	1–10	10–10^2	10^2–10^3	10^3–10^4	10^4–10^5	10^5–10^6	10^6–10^7	10^7–10^8	$>10^8$ organisms/ml	Total
0	96										96
0–10	1										1
1–10	17										17
10–10^3	26		1	2							29
10^3–10^4	21		1	1							23
10^4–10^5	17			1	1						19
	178										
10^5–10^6	6					1					7
10^6–10^7	5										5
10^7–10^8								1		1	2
$>10^8$ organisms/ml	6							1	1	8	16
	17										
Total	195			4	1	1		2	1	9	215

FROM THE DEPARTMENT OF MEDICAL MICROBIOLOGY, UNIVERSITY OF TURKU, FINLAND (HEAD PROFESSOR EERO MUSTAKALLIO M.D.) AND THE MUNICIPAL MATERNITY HOSPITAL OF TURKU (HEAD ROBERT HASSELBLATT, M.D.)

COLONY COUNT FROM MID-STREAM VOIDED URINE SPECIMENS AS A SCREENING METHOD FOR BACTERIURIA IN PREGNANT FEMALES[1]

By

SIMO VIRTANEN

Received 16.1.62

According to *Kass* (5, 7), true bacteriuria is seen in 6 per cent of the women in medical outpatient departments and of pregnant women. From a Finnish series, *Virtanen & Kasanen* (13) found asymptomatic bacteriuria in 5 per cent of the women in the medical ward and *Virtanen* (12) in 5.3 per cent of pregnant women at term.

Catheterization, however, involves a certain risk of infection, usually estimated at 2–4 per cent (4, 6, 7). Certain groups of patients are particularly susceptible to urinary tract infections, for instance, patients with urinary tract obstructions or anomalies, diabetics and patients with disturbance of bladder function (1, 8). Catheterization is considered the paramount risk of introducing urinary infection into susceptible persons (4). The risk of infection associated with catheterization is unusually high in diabetics (2). According to *Roberts* (11), the risk of catheterization in diabetics approximates that of thyroidectomy. Even those who believe that a catheter properly used can be employed safely without danger of introducing infection into patients who are voiding satisfactorily hold that in cases of debilitated patients and patients who are not voiding effectively catheterization can lead to serious, urinary infection (10).

Ureteral stasis and dilatation during pregnancy predispose to urinary tract infection (3, 9, 14). For this reason, pregnant women should be classified as particularly susceptible to urinary tract infection. catheterization should be made from a mid stream voided specimen.

Mid-stream voided and catheterized specimens from the female patients of a medical ward were compared by *Virtanen & Kasanen* (13) who found that one mid stream voided specimen was 84 per cent reliable. However, since pregnant women constitute a special group, ana-

[1]Supported in part by a grant from the *Rockefeller Foundation* and in part by a grant from the *State Commission for Medical Science*.

though the catheterized specimen was completely negative In 17 of these cases, 8 7 per cent, there were over 100,000 organisms/ml In a total of 8 4 (± 2 0) per cent of the cases which on the basis of the catheterized specimen could not be classified as significant bacteriuria, the mid stream specimens showed > 100,000 organisms/ml One mid-stream voided specimen, therefore, can be considered about 90 per cent reliable

As can be seen from Table 3, E coli was responsible for the bacteriuria, except in one case in which a closely related Citrobacter was found Also in the mid-stream voided specimen, E coli was the usual contaminant (Table 2) The closely related enterobacteria, E coli, Citrobacter and Klebsiella, comprised 46 4 per cent of the contaminants isolated

TABLE 3

Strains Isolated from Urine Specimens in 13 Cases of "True Bacteriuria"

Strain	Catheterized specimen		Mid-stream voided specimen		
	Alone	With one other strain	Alone	With one other strain	With two other strains
E coli	11	1	10	1	1
Citrobacter	1			1	–
Achromobacter	–	–	–		1
Staphylococcus albus		–	–	1	
Enterococci		–		–	1
Candida	–	1		1	
Total cases	12	2/2=1	10	4/2=2	3/3=1

DISCUSSION

Compared with the investigation on women in the medical ward (13), which gave false positive results in 16 (± 2 9) per cent of the mid-stream voided specimens, the series of parturients gave fewer, false positive results *viz* 8 4 (± 2 0) *per cent* The difference is statistically significant (0 05 > P > 0 02) Completely negative *cultures of mid-*stream voided specimens were obtained in 44 7 (± 3 4) per cent of the parturients, as against only 33 3 (± 2 7) per cent in the medical ward series This difference is *statistically highly* significant (P < 0 001) Therefore contamination associated with the drawing of the sample seemed to be a less frequent occurrence in parturients

Vaginal discharge being increased during pregnancy, the opposite result might have been *expected* Probably, the reason for the lower contamination rate in parturients is the more thorough cleansing of the external genitals before specimens *are taken* As the parturients were much younger (their mean age being lower by 50 per cent as compared with age in the medical series), better cooperation of the patient and

tomically as well as physiologically, the comparison between mid stream voided and catheterized urine specimens was carried out in the following series

SERIES AND METHODS

The series comprised 215 parturients of Turku Municipal Maternity Hospital Their mean age was 25 4 ± 0 4 years, 50 9 per cent were primiparas and 49 1 per cent multiparas Of the parturients without rupture of membranes admitted to the Hospital consecutively only those whose medical history did not indicate a presence of urinary tract infection were selected for the series

After the routine preparatory measures described in detail in an earlier paper (12) the parturient was asked to pass urine without voiding the bladder completely The specimen was taken from mid stream directly into a sterile test tube Subsequently the patient was catheterized by the techniques described previously (12) The treatment of the urine specimens colony count and bacterial identification were effected as described earlier (13)

RESULTS

The results are shown in Table 1 The limits for significant bacteriuria > 10,000 organisms/ml of urine in the catheterized specimen and > 100,000 organisms/ml of the mid stream voided specimen are indicated by the wider vertical and horizontal spaces in the table

TABLE 2

*Strains Isolated from Mid Stream Voided Urine Specimens in 106 Cases of Non Significant Bacteriuria**

Strain	Alone	With one other strain	Total strains
E coli	33	11	44
Citrobacter	6		6
Klebsiella	6	3	9
Proteus	2		2
Achromobacter	7	2	9
Alcaligenes faecalis	1	1	2
Corynebacterium xerosis	1		1
Lactobacilli	1	1	2
Staphylococcus albus	5	6	11
Staphylococcus citreus	2		2
Sarcina	1		1
Streptococcus α hemolyticus	4	5	9
Enterococci	6	5	11
Candida	11	6	17
Total cases	86	40/2=20	126

* <10000 organisms/ml in the catheterized urine specimen

Significant bacteriuria was found in 13 catheterized specimens 6 per cent

Both specimens were completely negative in 96 cases, 44 7 per cent In 99 cases, growth was seen in the mid stream voided specimen al

Co., Philadelphia and London 1947

superior personal hygiene may also have influenced the result Obviously, careful cleansing of the external genitals is important when mid stream voided specimens are taken

According to Kass (7), the confidence limit of a single bacterial count is about 95 per cent as regards catheterized specimens and about 80 per cent as regards voided specimens An 90 per cent confidence limit from a mid-stream voided urine specimen, therefore, must be considered quite good One of the drawbacks of mid-stream voided urine specimens mentioned previously (13) is that in cases of "true bacteriuria' multiple organisms may be found in the culture as a result of contamination

Bacteria may increase in the urine at a high rate at room temperature even For this reason, the specimen must be cultured without *delay or* kept in cold storage until cultured This restricts the use of mid-stream voided specimens to hospitals with a bacteriological laboratory of their own, and to places with an immediately accessible laboratory service A cooled specimen can be kept for examination for two days (5)

If the storage and transport of cooled specimens is organized the method can be employed in smaller hospitals and maternity welfare centres as well

SUMMARY

Colony counts of both mid-stream voided and catheterized urine specimens were performed for 215 parturients on admission to maternity hospital Compared with the catheterized specimen, the mid stream voided specimen had a confidence limit of 90 per cent

The importance of careful cleansing of the external genitals before taking the specimen and of immediate culture or cooling of the specimens is emphasized

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DEMONSTRATION OF OXIDATIVE ENZYMES IN THE HUMAN PANCREAS WITH SPECIAL REFERENCE TO THE ISLETS OF LANGERHANS

By

CLAES HELLERSTRÖM and BO HELLMAN

Received 12 xii 61

It has been suggested that the regeneration of TPNH in connection with glucose breakdown via the HMP shunt might be of great importance for the synthesis and release of insulin (*Field, Johnson, Herring & Weinberg* 1960, *Lazarus & Bradshaw* 1959, *Hellman & Hellerström* 1962) High activities of enzymes generally associated with an active HMP pathway were demonstrated in the islet B cells from rabbits and mice (*Lazarus & Bradshaw* 1959, *Hellman & Hellerström* 1962), and *Field, Johnson Herring & Weinberg* (1960), after incubating slices of a human B cell tumour, reported 5 6 times more radioactive CO_2 in the presence of glucose-1-^{14}C than with glucose-6-^{14}C The absence, hitherto of any data concerning the metabolic pathways for glucose in normal human islet tissue may be ascribed not only to the limited possibilities of obtaining fresh material but also to difficulties in applying biochemical techniques to such a diffusely scattered organ, which comprises only 1 2 per cent of the volume of the whole pancreas (*Hellman* 1959) A histochemical approach to this question, however became possible with the introduction of methods for the

Our sincere thanks are due to Professor *O Hultén* M D., Surgical Clinic of the University Hospital Uppsala who kindly supplied us with pancreatic material

The following abbreviations are used

DPN — [illegible]
DPND — [illegible]
DPNH — [illegible]
TM — [illegible]
G 6 PD — [illegible]
HMP — [illegible]
LD — [illegible]
MTT — [illegible]
Nitr BT — [illegible]
PVP — Polyvinyl pyrrolidone (M W 11000)
SD — Succinic dehydrogenase
TPN — [illegible]

BRIEF REPORT

THE FREQUENCY OF THE DUFFY BLOOD GROUP FACTOR Fy^a IN SWEDEN

By *Aage Heiken*

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m

Sanger et al (1955) since they observed that a great number of blood samples from Negroes reacted negatively to both anti Fy^a and anti Fy^b (demonstrated by *Ikin et al* 1951) However while the *Fy* gene frequency in certain Negroid populations amounts to about 90 per cent the gene is absent or at least extremely rare in Caucasians (*Race & Sanger* 1958)

Although the Duffy blood group system thus seems to be of great interest from an anthropological point of view the geographical distribution of the Duffy alleles has been only sporadically investigated probably because of the scarcity of testsera Since no frequencies have so far been published for the Swedish population it was found of interest to summarize the data obtained at the State Institute for Blood Group Serology in connection with paternity cases

In the last year blood samples from 344 adult Swedes from the entire country were tested with anti Fy^a sera The indirect anti globulin technique was used and all sera were checked by means of known testcells 299 persons were found to give a positive reaction On the basis of this result the following figures can be calculated given that the Fy(a—) phenotype really represents the Fy^bFy^b genotype

Phenotypes per cent		Genotypes per cent			Genes per cent	
Fy(a+)	Fy(a—)	Fy^aFy^a	Fy^aFy^b	Fy^bFy^b	Fy^a	Fy^b
66 57	33 43	17 79	48 78	33 43	42 18	57 82

Control of the genetic composition of the mate [illegible] gene frequency given above corresponds closely to that (*Fy^a* = 41 91 per cent) by *Gürtler* (1962) and found in Great Britain (cf Mourant 1954) Howe[illegible] the *Fy^a* gene frequencies which according to *Allison et al* (1956) have been found among both Swedish (*Fy^a* = 55 28 per cent) and Norwegian (*Fy^a = 81 89 per cent*) Lapps

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Received 23 v 62 *from the State Institute for Blood Group Serology Stockholm (Head Professor Birger Broman M D)*

C The TPND and DPND media (*Scarpelli Hess & Pearse* 1958) consisted of a

potassium salt 1 0 M) 0 1 ml TPN (0 1
nl MgCl (0 05 M) 0 25 ml tris maleate
mg/ml) 0 05 ml $CoCl_2$ (0 5 M) 0 05 ml

Conditions of incubation The cover slips were placed in prewarmed (37° C) plexiglass chambers (*Hellman & Hellerstrom* 1962) and the sections covered with 0 2–0 4 ml of the substrate m
at 37° C until the appr pri
15–25 minutes for DPND an
Control sections as well we
reaction was never revealed ... for 30 seconds and fixation in 10 per cent form 1 sal ne for 15 minutes the sections were mounted in glycerine jelly c nta ning 0 05 M CoCl The incubation in the Nitro BT containing LD medium was followed instead by rinsing in distilled water fixation in formol saline and mounting in pla n glycerine jelly

RESULTS

Examination in dark field illumination of the fresh cryostat sections of the human pancreas showed that the islets had a strong yellowish white luminescence which made them clearly recognizable against the darker exocrine parenchyma (Fig 1) This was caused by a fine cytoplasmic granulation more concentrated in the capillary poles which revealed the islet cell nuclei as dark spots In thin sections (not more than 2 3 μ thick) some islet cells had a more silvery white granulation with a narrow perinuclear distribution The acinar cells had a very faint yellowish tinge while the connective tissue surrounding excretory ducts and blood vessels exhibited a slightly stronger white luminescence



Fig 1

Pancreatic islet in dark field illumination The cytoplasm of the islet cells is filled with bright luminescent granules and some capillaries can be seen as darker spots Surr unding exocrine parenchyma almost completely dark × 400

demonstration of oxidative enzymes in tissue sections by means of suitable tetrazolium salts (*Farber, Sternberg & Dunlap* 1956, *Nachlas, Tsou, de Souza, Cheng & Seligman* 1957, *Scarpelli, Hess & Pearse* 1958 *Hess, Scarpelli & Pearse* 1958, *Pearse* 1957) These compounds are reduced to coloured insoluble formazans by electron transfer from enzymes involved in the cell respiratory chain (*Zimmermann & Platt* 1960, *Pearse* 1960, *Schumacher* 1961) and in this way reflect the enzyme activity in that particular part of the cell metabolism which is associated with the utilization of glucose

The present investigation deals with an application of these histochemical methods in a study of enzymes concerned with the oxidative metabolism in the islet tissue and the exocrine pancreatic parenchyma in man The following enzymes in the different pathways of glucose metabolism were chosen G-6-PD in the HMP shunt, LD in the EM pathway and SD in the Krebs cycle In addition the TPND and DPND activities were studied in the different parts of the human pancreas

MATERIAL AND METHODS

Preparation of tissue Pancreatic tissue was obtained from five male patients suffering from gastric or duodenal ulcers The patients were from 30 to 73 years old without any signs of diabetes and in good preoperative condition During the gastric resection a pea sized piece of the pancreatic tail was removed and rapidly divided into slices 2–3 mm thick which were placed on thin strips of aluminium foil These were plunged into a beaker containing isopentane cooled to —70° C with a dry ice alcohol mixture The frozen tissue was sectioned immediately in a cryostat (*Pearse Slee*) or stored at —70° C in small stoppered test tubes

Sections 5 10 µ thick were attached to cover slips by warming the under surface of the glass with a finger The first sections obtained from a tissue block were mounted in glycerine jelly and immediately examined in dark field illumination (Leitz bicentric immersion dark field condensor D 1 20 A) In this way rapid information was obtained about the presence and general distribution of the islet tissue which could be clearly distinguished from the exocrine parenchyma by its strong luminescence (*cf Logothetopoulos & Salter* 1960 *Hellman Hellerstrom Larsson & Brolin* 1961) When islet tissue had been found every alternate serial section was taken for the demonstration of oxidative enzymes The remaining sections were silver impregnated as described by *Hellerstrom & Hellman* (1960) to simplify the exact localization of the islets in adjacent sections stained for enzyme activity Good silver impregnation results were obtained in sections postfixed in 6 per cent calcium acetate formalin for 15 minutes followed by Bouin's solution for about 4 hours at room temperature After the subsequent silver impregnation procedure the islets could be clearly identified by the distinct blackening of some of their cells

Composition of substrate solutions All the media were prepared immediately before use The reagents including the tetrazolium salts (MTT and Nitro BT) were obtained from the Sigma Chem Comp St Louis 18 Mo USA

After attachment to cover slips the unfixed cryostat sections were incubated in solutions of the following compositions

A The solution for SD (*Pearse* 1960) consisted of 2 5 ml phosphate buffer at pH 7 4 (0 06 M), 0 3 ml $CoCl_2$ (0 5 M) 2 5 ml MTT (1 mg/ml), 3 0 ml sodium succinate (0 2 M) distilled water to 10 0 ml and 750 mg PVP The pH was adjusted to a final value of 7 0 with stock tris buffer of pH 10 4 (0 2 M)

B The LD medium (*Hess Scarpelli & Pearse* 1958) consisted of 0 1 ml sodium DL lactate (1 0 M), 0 1 ml DPN (0 1 M) 0 1 ml sodium cyanide (0 1 M), 0 1 ml $MgCl_2$ (0 05 M), 0 1 ml distilled water 0 25 ml tris maleate buffer at pH 6 8 (0 2 M) 0 25 ml Nitro BT (1 mg/ml) and 75 mg PVP



Fig 6

Distribution of G 6 PD in the pancreas The enzyme activity is high in the islet tissue (central part of the picture) while the reaction in surrounding acinar cells is rather weak × 350

There was a pronounced enzymatic reaction for *SD* in the exocrine cells the cytoplasm of which was densely packed with black dots of cobalt formazan (Fig 2) On the whole the duct epithelia had the same activity as the acinar cells An interlobular duct with positively reacting epithelial cells can be seen in Fig 7 A From this figure it is also evident that the periductal connective tissue appeared devoid of activity As compared to the exocrine parenchyma the islets could be identified as areas with lower enzyme activity (Fig 2) While most of the islet cells contained scattered formazan deposits mainly with a perinuclear cytoplasmic distribution there were on the other hand some groups of cells which did not show any reaction at all

Presence of *ID* activity was found throughout the pancreas In the exocrine parenchyma the formazan pattern of the Nitro BT tetrazolium consisted of dark blue dots or rods and very fine blue violet grains The coarse dark blue precipitate was distributed mainly in the central part of the acini (Fig 3) In the islets of Langerhans the ID activity was

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Figs 2 5

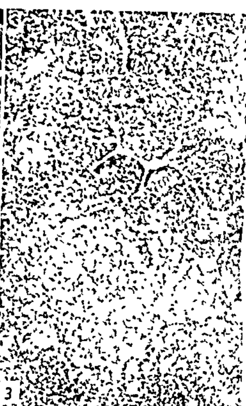
Fig 2 SD activity in the pancreatic tissue The rather strong reaction in the exocrine parenchyma contrasts against the lighter islet tissue in the lower central part of the picture × 340

Fig 3 The distribution of ID activity in the pancreatic tissue using Nitro BT as an electron acceptor In the lower part of the picture is an islet with a slightly weaker activity than the surrounding exocrine parenchyma × 200

Fig 4 DPND activity in the pancreas In the central part of the picture an islet may be seen with about the same activity as the surrounding exocrine parenchyma × 200

Fig 5 Distribution of TPND in the pancreatic tissue The central part of the picture shows a large islet with about the same enzyme activity as that of the surrounding exocrine parenchyma × 250


2


3



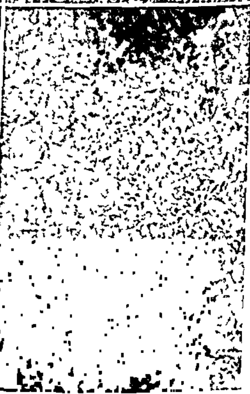




Fig 6

Distribution of G 6 PD in the pancreas The enzyme activity is high in the islet tissue (central part of the picture) while the reaction in surrounding acinar cells is rather weak × 350

There was a pronounced enzymatic reaction for *SD* in the exocrine cells, the cytoplasm of which was densely packed with black dots of cobalt formazan (Fig 2) On the whole the duct epithelia had the same activity as the acinar cells An interlobular duct with positively reacting epithelial cells can be seen in Fig 7 A From this figure it is also evident that the periductal connective tissue appeared devoid of activity As compared to the exocrine parenchyma the islets could be identified as areas with lower enzyme activity (Fig 2) While most of the islet cells contained scattered formazan deposits, mainly with a perinuclear cytoplasmic distribution, there were on the other hand some groups of cells which did not show any reaction at all

Presence of *LD* activity was found throughout the pancreas In the exocrine parenchyma the formazan pattern of the Nitro BT tetrazolium consisted of dark blue dots or rods and very fine blue violet grains The coarse dark blue precipitate was distributed mainly in the central part of the acini (Fig 3) In the islets of Langerhans the LD activity was

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Figs 2 5

Fig 2 SD activity in the pancreatic tissue The rather strong reaction in the exocrine parenchyma contrasts against the lighter islet tissue in the lower central part of the picture × 340

Fig 3 The [illegible] elect [illegible] weal [illegible]

Fig 4 DPN [illegible] pancreas In the central part of the picture an islet may be seen with about the same activity as the surrounding exocrine parenchyma × 200

Fig 5 Distribution of TPND in the pancreatic tissue The central part of the picture shows a large islet with about the same enzyme activity as that of the surrounding exocrine parenchyma × 250



less pronounced. The reaction product in most of the islet cells consisted nearly exclusively of the coarser precipitate with a perinuclear distribution. It was noted that a smaller fraction of the islet cells lacked such activity. The reaction for ID was considerable in the interlobular duct epithelium but for the ductules the reaction was of about the same degree as in the acinar cells (Fig. 7 B).

Figs 7–8

Fig 7 A) SD reaction in interlobular duct epithelium Surrounding connective tissue lacks demonstrable activity while the acinar tissue shows approximately the same degree of reaction as the epithelial cells × 250
B) Distribution of LD activity in epithelial cells from a large interlobular duct using Nitro-BT as an electron acceptor Epithelial cell nuclei are surrounded by discrete formazan dots To the left negatively reacting connective tissue Duct lumen to the right × 1325
[illegible] in the

Fig 8 [illegible] part of [illegible] iductal [illegible] he left

B) Epithelial cells of a ductule showing stronger TPND activity than surrounding exocrine parenchyma × 1300
C) G 6 PD activity in ductular epithelium Surrounding exocrine parenchyma shows moderate activity × 925

The reaction for *DPND* was moderate both in the acinar cells (Fig 4) and in the interlobular duct epithelium and marked for the epithelial cells lining the smaller ductules (Fig 7 C) As can be seen in Fig 4, the enzyme activity of the islet tissue did not differ appreciably from that of the acinar cells It was therefore possible to distinguish the islets clearly from the exocrine parenchyma only after comparison with adjacent silver impregnated sections

The distribution and reaction intensity for *TPND* was about the same as for DPND (Fig 5) Thus among the moderately reacting acinar cells the ductular epithelium could be recognized on account of its more intense staining reaction (Fig 8 B) However, the interlobular duct epithelium also showed a rather strong reaction, which was mainly confined to the supranuclear luminal pole of the cells (Fig 8 A) No signs of obvious differences in enzyme activity were noted between the islet cells

The reaction for *G-6-PD* in the islet tissue and the surrounding exocrine parenchyma is illustrated in Fig 6 The enzyme activity was most pronounced in the islet cells, where the cobalt formazan dots were characteristically situated in the perinuclear region of the cytoplasm Even when using prolonged incubation times some cells in the islets remained unstained, and only a moderate reaction was noted in the acinar tissue In the exocrine part of the gland the highest activity was found in the ductular epithelium (Fig 8 C)

DISCUSSION

The basic principles for the demonstration of oxidative enzymes in tissue sections by means of tetrazolium salts have recently been reviewed by *Pearse* (1960) Under appropriate histochemical conditions it is possible by using some of these salts, especially MTT and Nitro-BT, not

only to demonstrate accurately the localisation of tissue enzymes but very probably also to get a rough estimate of the relative activities of an enzyme in different structures. It should be pointed out, however, that control experiments are necessary, since unspecific reduction to formazan may occur. Such a nonenzymatic staining may for example appear, if the pH of the incubation medium is within the alkaline range, this is probably due to the action of protein-bound SH-groups (*Pearse & Zimmermann* 1959, *Zimmermann* 1961). In the present investigation the pH of the incubation solutions was consequently kept at 6.8–7.2 the higher value used for the diaphorases, since their substrates TPNH and DPNH, are rapidly destroyed in the acid range (*Pearse* 1960). The absence of any staining in the sections, which were incubated in substrate-free media, strongly supports the view that the distribution of formazan observed in the human pancreas reflects real enzyme activity.

It cannot be excluded that the anesthesia during surgery might have had some influence on the enzyme pattern observed in the pancreas. It is known that barbiturates in high concentrations have a blocking effect on cell respiration. This property of the barbiturates has also been used in the present investigation for increasing the rate of reduction of tetrazolium salts in the TPND, DPND and G–6–PD reactions (*cf. Pearse* 1960). However, the small barbiturate concentrations obtained *in vivo* in connection with surgical anesthesia have been reported to have only an insignificant depressive effect on the oxygen uptake of liver and brain (*Brody & Bain* 1951).

It was obvious that the oxidative enzymes were not equally distributed between the endocrine and exocrine parts of the pancreas. Thus, the reactions for SD and LD were more pronounced in the acinar cells than in the islet tissue, whereas the opposite was found for G–6–PD. This latter enzyme is concerned with the conversion of glucose–6–phosphate into 6-phosphogluconate, which is the first step in glucose breakdown via the HMP shunt. Consequently the high activity of G–6–PD has been interpreted as indicating the existence of an actively operating HMP shunt also in normal human islet tissue.

The distribution of oxidative enzymes has been analyzed previously in the pancreas of normal and obese-hyperglycemic mice (*Hellman & Hellerström* 1962). These results were compared with the human enzyme pattern by incubating pancreatic sections from the two species in the same batches of substrate solutions for equal periods of time. The human islets showed weaker reactions for G–6–PD and TPND, approximately equal staining intensities for SD and DPND and a stronger reaction for LD. The biological significance of these species differences is still obscure. Signs of a more active HMP shunt in the mice (particularly strong reaction for G–6–PD) might reflect a higher capacity for insulin synthesis than in man. It is also possible that this metabolic feature has some relation to the great regenerative power of the islet tissue in rodents, exemplified by the spontaneous recovery from alloxan

diabetes (*Lazarow* 1952) and in a tenfold increase of the total islet volume in obese hyperglycemic mice (*Gepts, Christophe & Mayer* 1960, *Hellman, Brolin, Hellerström & Hellman* 1961).

The human islet tissue is composed of about $^2/_3$ B cells and $^1/_3$ A cells (*Gepts* 1957). Since positive enzyme reactions could be demonstrated in the overwhelming majority of the islet cells, our findings must be representative for the B cells at least. The occurrence of islet structures *having no obvious* enzyme activity in connection with the dehydrogenase reactions can probably be explained by the presence of connective tissue, but may also be due in part to A₁ or A₂ cells (*cf. Hellman & Hellerström* 1961). Differences in the enzyme pattern between A and B cells have been found in rabbits (*Lazarus & Bradshaw* 1959) and in ducks (*Hellerström*, to be published), where these cells can be identified directly in sections stained for oxidative enzymes.

The enzyme activity was especially intensive in the epithelial cells of ductules and interlobular ducts. *Stier* (1952) found a strong reduction of tetrazolium in these cells after intravenous injection of triphenyl tetrazolium chloride (TTC) to guinea pigs. Furthermore *Lazarus & Bradshaw* (1959) noted relatively strong reactions for G-6-PD, TPND, LD, DPND and SD in the pancreatic duct epithelium of rabbits. The high enzyme activities of these structures are rather surprising, since no convincing evidence exists that they have any secretory function.

SUMMARY

Histochemical methods were used to demonstrate the following oxidative enzymes in the human pancreas: glucose-6-phosphate dehydrogenase (G-6-PD), lactic dehydrogenase (LD), succinic dehydrogenase (SD) [illegible]

[illegible] reactions for LD and SD were found to be weaker and for TPND and DPND of about the same order as compared to the exocrine parenchyma. With the exception of SD especially intense enzyme activity occurred in the epithelium cells of the excretory duct system. The occurrence of some islet structures with no obvious dehydrogenase enzyme activities might in part be ascribed to A cells.

In analogy with previous histochemical observations in mice and rabbits the results have been interpreted as indicating the existence of an actively operating hexose monophosphate sh[illegible]

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AMYLOID "TUMOUR" IN SIGMOID COLON

By

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Received 27 i 62

Amyloid deposits are common in the digestive tract in primary (atypical) amyloidosis (for surveys *see* *Koletsky Stecher* 1939, *Eisen* 1946, *Dahlin* 1949, *Matthews* 1954, *Korelitz-Spindell* 1956, *Symmers* 1956) In primary amyloidosis with circumscribed amyloid deposits in the gastro-intestinal tract, amyloid degeneration is, as a rule also demonstrable in other organs, and only one case of amyloidosis of the digestive tract without co-existing amyloid changes elsewhere has been described (*Intriere Brown* 1956) The following report of a case of amyloid "tumour" in the sigmoid colon without signs of amyloidosis elsewhere was therefore considered legitimate

REPORT OF CASE

[illegible]
[illegible] on check radiographs 2 years later the polyp was found to [illegible] at the
have increased in size it now projected 2 5 cm into the lumen of the intestine, its
surface being irregular The intestinal lumen was narrowed and it was suspected
that the polyp h[illegible]
[illegible] stro intestinal symp
[illegible] nothing remarkable
[illegible] he electrocardiogram
[illegible] was subjected to operation with resection
of 13 cm of the sigmoid colon and end to end anastomosis The post operative
course was smooth

Gross examination of the operative specimen showed a polypoid mass bulging into the lumen The mass was twice the size of a brown bean it was of firm consistency and had a broad base (Fig 1) Its surface appeared to be covered with mucosa and the cut surface was homogeneous and greyish white to greyish-yellow Immediately distal to the mass was a shallow diverticulum as the only other gross change in the specimen The intestinal wall was of normal thickness and consistency and its mucosa was of normal mobility

Histological examination showed the stroma of the polyp to be made up of short, plump, structureless trabeculae Most of these trabeculae were separated from one another by connective tissue, but in some parts they formed homogeneous, roundish formations containing calcigerous deposits and [illegible]d by thin connective tissue lamellae



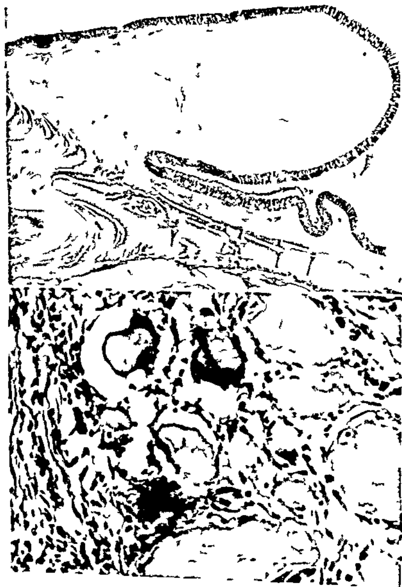
Fig 1
Well-defined polypoid tumour in sigmoid colon

(Fig 2) Small focal and streaky infiltrates of round cells and single multinucleated giant cells of foreign body type were seen in the structureless amorphous masses, particularly in their periphery (Fig 3) The surface was covered with atrophic mucosa with normal glands, and sparse deposits of round cells It showed no signs of ulceration or bleeding The muscularis mucosa and the inner layer of the muscularis propria were partly replaced by the structureless masses, which also followed the floor of the diverticulum The surface of the diverticulum facing the subserous fatty tissue thus had no muscularis propria but was instead enveloped by a mass of irregularly arranged structureless trabeculae, which bulged into the subserous tissue (Fig 4) No pathological changes could be seen elsewhere in the operative specimen

The structureless masses stained pale-pink with haematoxylin and eosin, and orange with v Gieson's stain Staining with methyl violet and Congo red gave the colour reaction typical of amyloid, but the metachromasia and the intensity of the colour varied from one part to another The cytoplasm in the multinucleated giant cells also stained metachromatically with methyl violet In the sections stained for elastica (according to Weigert) the substance assumed a pale brown-red colour The substance often contained traces of vessels with tiny residues of elastic lamellae in rounded, structureless mantles around a central lumen lined with endothelium

F g 2

The polypo l tumour of s gmo d colon l ned with mucosa The stroma cons sts of h rt structureless trabeculae often gathered in homogeneous rounded formations × 7 Htx eos n



F g 3

C llect n f l mph cytes an l multinucleated giant cells in the stroma The g ant cells c ntain an amorph us sul stance × 300 v Gieson



Fig 4
Diverticulum in sigmoid colon The diverticulum is enveloped by an amorphous mass partly collected in rounded formations × 15 v Gieson

DISCUSSION

The term primary amyloidosis is used to designate cases of amyloidosis in patients without predisposing disease In primary amyloidosis amyloid deposits are seen in the main in mesodermal tissues in organs rarely affected by the secondary (typical) amyloidosis In primary amyloidosis the gastro-intestinal tract is often affected, the stomach being the most common site of the changes Amyloid deposits occur, above all, in and around vessels in the submucosa, but all the coats of the stomach and bowel can be affected The symptoms of gastro intestinal amyloidosis are not characteristic, and the true nature of the disease often remains concealed until post-mortem examination Amyloid deposits in the submucosa are often focal and tend to form well defined circumscribed solitary or multiple "tumours" (*v Bonsdorff* 1933) They may cause mechanical obstruction with ileus as a result, or they may give rise to mucosal ulceration with consequent intestinal haemorrhage (*Steinhaus* 1902, *Konigstein* 1925 *Lubarsch* 1929, *Gerstel* 1932, *Randall* 1933, *Michelson-Lynch* 1934, *Golden* 1945, *Golden* 1954, *Symmers* 1956) The clinical picture of gastric amyloidosis may simulate that of cancer of the stomach (*Steinhaus* 1902, *Lubarsch* 1929, *Williams-Baggenstoss* 1952, *Shnider-Burka* 1955 *Intriere-Brown* 1956, *Klingenberg* 1958)

In the present case the pre-operative diagnosis was polypoid tumour

of the sigmoid colon probably becoming malignant The site of the tumour as well as its histological structure and tinctorial properties fitted in with a diagnosis of primary amyloidosis of the gastro intestinal tract There was no clinical evidence of any predisposing disease and no clinical signs of amyloidosis elsewhere Neither did the further investigation reveal any evidence for multiple myeloma as a cause of the amyloid deposits—a possibility that must be considered because of the close similarity between the distribution of local amyloid foci in the gastro intestinal tract in multiple myeloma and primary amyloidosis (see e g *Magnus Levy* 1931 *Dahlin Dockerty* 1950 *Korelitz Spindell* 1956) Finally the possibility of a local diverticulitis in the sigmoid colon as a cause of amyloid deposits in surrounding tissues cannot be excluded The inflammatory changes however were slight only and were regarded as secondary to the amyloid deposits Therefore it appears less likely that the amyloid "tumour in the present case was due to diverticulitis

SUMMARY

A case of a tumour like amyloid deposit in the sigmoid colon in a 66-year old woman is reported The pre operative roentgenography showed a polypoid tumour of the sigmoid colon which presumably was becoming malignant Histologically the tumour was built up of structureless masses with the tinctorial properties of amyloid The patient was followed up for three years but never showed any other signs of amyloidosis

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THE PANCREATIC ISLET TISSUE IN MICE WITH OBESITY INDUCED BY GOLDTHIOGLUCOSE

By

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Received 19 III 62

Distinction has been made between the "regulatory" obesity, in which the primary impairment lies in the central mechanisms regulating food intake, and "metabolic" obesity, in which the mechanism is an inborn or acquired metabolic defect of the tissues (cf *Mayer* 1960) Concerning the "metabolic" type there is a great deal of information about the pancreatic islet tissue in the American variety of the obese-hyperglycaemic syndrome in mice These obese mice (AO mice) have a striking hyperplasia of the islet tissue (*Gepts, Christophe & Mayer* 1960, *Hellman, Brolin, Hellerstrom & Hellman* 1961), signs of increased B cell activity (*Hellman & Petersson* 1960) and a reduced proportion of the argyrophil type of A cells (*Hellman* 1961) in comparison with their lean litter mates (AN mice) It has been shown that the appearance of the islets was closely associated with the amount of food ingested but was on the other hand, not completely normalized by maintaining the animals within a normal body weight range (*Petersson & Hellman* 1962)

The present work deals with the reactions of the pancreatic islet tissue in "regulatory" [illegible]
The use of AN mice [illegible]
comparison with pre[illegible] obesity in
the AO mice

MATERIAL AND METHODS

Male mice belonging to the obese [illegible]
[illegible] eceived a single
[illegible] goldthioglucose (*Solganal B*
von B Schering AG., Berlin)

The surviving animals were killed by extension of the neck about 8 months after the goldthioglucose injection during the last month they were given 50 per cent more fat in the diet to increase the obesity (cf *Marshall & Mayer* 1954) They were classified into two groups

1. Twelve lean mice (body weight 29.2 ± 0.4 g) referred to as AN-mice
2. *Ten mice with considerable weight gain (body weight 44.4 ± 1.3 g) exhibiting* "regulatory" obesity and referred to as ANO-mice

The pancreas was weighed after 24 hours' fixation in Bouin's solution. The histological treatment of the organ was performed in a uniform manner throughout according to the scheme used previously (*Hellman* 1959). The paraffin embedded *organs were sectioned serially. In addition* to the silver impregnation procedure described by *Hellerström* & *Hellman* (1960) the granule staining methods with victoria blue according to *Ivić* (1959) and aldehydefuchsin ponceaufuchsin were used.

After staining with victoria blue sections at regular intervals (the distance between the sections was 154 µ) were scanned systematically for islet section surfaces at a magnification of 125 ×. Only those islet section surfaces whose area equalled or exceeded that of a standard circle 110 µ in diameter were counted. This number was used to compute the total islet volume according to the method of *Hellman* (1959) and using the data obtained for the size frequency distribution of the islet tissue in this strain of mice (*Hellman, Brolin, Hellerström* & *Hellman* 1961). The determinations of the total islet volume were made in duplicate, where each single determination was based on the total number of islet section surfaces counted in every alternate section used for scanning analysis. The error for a single determination was calculated from the difference between the duplicates as about ± 10 per cent in the AN-group and ± 4 per cent in the ANO group.

The frequency of silver positive islet cells was determined in 4 µ thick sections in which the impregnation results were assessed as satisfactory. The islets were included in the order in which they were encountered, at least 1000 cells being counted from a minimum of 20 islet section surfaces in each animal (magnification 510 ×). To obtain an approximate correction for the systematic error due to the inequalities in size of the cells and nuclei counted, determinations were made also of the diameters of the whole cells, nuclei and nuclear fragments by means of an ocular screw micrometer (cf. *Hellman* 1959, *Hellman* & *Hellerstrom* 1961). The random error for each determination of the frequency of silver cells was less than ± 10 per cent.

RESULTS

The hyperphagia of the ANO-mice resulted in a significant higher pancreatic weight (see Tables 1 and 2). The values for these animals were 457 ± 24 mg compared with 345 ± 13 mg for the AN-mice (t = 4.34, P < 0.001).

Except for some hyperaemia there was no obvious difference in the islets of Langerhans of the ANO-mice as compared with the AN-*mice*. In both groups well granulated B cells were noted in *the sections* stained with aldehydefuchsin or victoria blue. Furthermore the intensity of the argyrophil reaction seemed to be of the same degree in adequately *impregnated sections*.

The results of the volumetric determination of the islet tissue have been presented in Tables 1 and 2. The total islet volume of the ANO-mice, 1.67 ± 0.21 mm³, was more than double that of the AN-mice 0.79 ± 0.04 mm³ (t = 4.48, P < 0.001). The percentile frequency of argyrophil islet cells was not changed in the goldthioglucose *induced* obesity. A value of 8.9 ± 0.6 per cent was found in the AN-group, while the corresponding figures for the silver-positive cells in the ANO-group was 8.0 ± 0.8 per cent (t = 0.94, P > 0.05).

TABLE 1

Weights of Body (g) and Pancreas (mg) and the Number of Islet Section Surfaces > 110 µ Counted (Columns I and II Give the Values for each of the Pair of a Duplicate Determination) in the Lean Mice The Total Islet Volume in mm³ Was Calculated from the Latter Values (see Section on Material and Methods) In addition to the Individual Figures the Mean Values and their Standard Errors Are Given at the Bottom of the Table

Animal No	Body weight (g)	Pancreas weight (mg)	Counted number of islet sections > 110 µ		Islet volume (mm³)
			I	II	
AN 1	28.8	388	49	63	0.67
AN 2	29.4	315	48	50	0.59
AN 3	27.0	318	64	59	0.73
AN 4	28.1	423	71	70	0.83
AN 5	27.8	384	82	74	0.92
AN 6	30.2	319	57	61	0.70
AN 7	28.6	334	55	55	0.66
AN 8	29.0	362	73	82	0.69
AN 9	28.4	258	68	68	0.80
AN 10	30.5	329	85	70	0.91
AN 11	31.2	366	79	99	1.04
AN 12	31.0	342	85	71	0.92
M ± S.E.	29.2 ±0.4	345 ±13			0.79 ±0.04

TABLE 2

Weights of Body (g) and Pancreas (mg) and the Number of Islet Section Surfaces > 110 µ Counted (Columns I and II Give the Values for each of the Pair of a Duplicate Determination) in the Goldthioglucose Obese Mice The Total Islet Volume in mm³ Was Calculated from the Latter Values (see Section on Material and Methods) In Addition to the Individual Figures the Mean Values and their Standard Errors Are Given at the Bottom of the Table

Animal No	Body weight (g)	Pancreas weight (mg)	Counted number of islet sections > 110 µ		Islet volume (mm³)
			I	II	
ANO 1	47.8	520	128	114	1.40
ANO 2	47.5	495	227	228	2.59
ANO 3	43.0	374	62	75	0.81
ANO 4	39.6	417	90	108	1.15
ANO 5	46.5	564	154	156	1.78
ANO 6	43.6	522	152	147	1.72
ANO 7	50.4	324	133	135	1.54
ANO 8	46.6	467	218	213	2.46
ANO 9	39.8	493	217	215	2.46
ANO 10	39.0	395	65	66	0.78
M ± S.E.	44.4 ±1.3	457 ±24			1.67 ±0.21

1 Twelve lean mice (body weight 29 2 ± 0 4 g) referred to as AN mice
2 Ten mice with considerable weight gain (body weight 44 4 ± 1 3 g) exhibiting 'regulatory' obesity and referred to as ANO mice

The pancreas was weighed after 24 hours' fixation in Bouin's solution The histological treatment of the organ was performed in a uniform manner throughout according to the scheme used previously (*Hellman* 1959) The paraffin embedded organs were sectioned serially In addition to the silver impregnation procedure described by *Hellerstrom* & *Hellman* (1960) the granule staining methods with victoria blue according to *Ivic* (1959) and aldehydefuchsin ponceaufuchsin were used

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The frequency of silver positive islet cells was determined in 4 μ thick sections in which the impregnation results were assessed as satisfactory The islets were included in the order in which they were encountered, at least 1000 cells being counted from a minimum of 20 islet section surfaces in each animal (magnification 510 ×) To obtain an approximate correction for the systematic error due to the inequalities in size of the cells and nuclei counted determinations were made also of the diameters of the whole cells nuclei and nuclear fragments by means of an ocular screw micrometer (cf *Hellman* 1959, *Hellman* & *Hellerström* 1961) The random error for each determination of the frequency of silver cells was less than ± 10 per cent

RESULTS

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Except for some hyperaemia there was no obvious difference in the islets of Langerhans of the ANO mice as compared with the AN mice In both groups well granulated B cells were noted in the sections stained with aldehydefuchsin or victoria blue Furthermore the intensity of the argyrophil reaction seemed to be of the same degree in adequately impregnated sections

The results of the volumetric determination of the islet tissue have been presented in Tables 1 and 2 The total islet volume of the ANO mice, 1 67 ± 0 21 mm³, was more than double that of the AN-mice, 0 79 ± 0 04 mm³ ($t = 4\ 48$ $P < 0\ 001$) The percentile frequency of argyrophil islet cells was not changed in the goldthioglucose induced obesity A value of 8 9 ± 0 6 per cent was found in the AN-group, while the corresponding figures for the silver positive cells in the ANO-group was 8 0 ± 0 8 per cent ($t = 0\ 94$, $P > 0\ 05$)

TABLE 1

Weights of Body (g) and Pancreas (mg) and the Number of Islet Section Surfaces > 110 μ Counted (Columns I and II Give the Values for each of the Pair of a [illegible]

Animal No	Body weight (g)	Pancreas weight (mg)	Counted number of islet sections 110 μ		Islet volume (mm³)
			I	II	
AN 1	28 8	388	49	63	0 67
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AN 7	28 6	334	55	55	0 66
AN 8	29 0	362	73	82	0 69
AN 9	28 4	258	68	68	0 80
AN 10	30 5	329	85	70	0 91
AN 11	31 2	366	79	93	1 04
AN 12	31 0	342	85	71	0 92
M +	29 2	345			0 79
S E	±0 4	±13			±0 04

TABLE 2

Weights of Bo [illegible] *l Section Surfaces > 11* [illegible] *of the Pair of a Dupl* [illegible] *Total Islet Volume in m* [illegible] *on Material and Metho* [illegible] *the Mean Values and their Standard Errors Are Given at the Bottom of the Table*

Animal No	Body weight (g)	Pancreas weight (mg)	Counted number of islet sections > 110 μ		Islet volume (mm³)
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ANO 8	46 6	467	218	213	2 46
ANO 9	39 8	493	217	215	2 46
ANO 10	39 0	395	65	66	0 78
M +	44 4	457			1 67
S E	±1.3	±24			±0 21

DISCUSSION

The obese-hyperglycemic syndrome in mice is characterized by an increase in the number of islets of Langerhans Recent studies have shown that the total islet volume in these animals is 8–10 times larger than in their lean litter mates (*Gepts, Christophe & Mayer* 1960, *Hellman, Brolin, Hellerstrom & Hellman* 1961) In spite of this, the relation between the total islet volume and the islet diameter is still mathematically symmetrical (*Hellman, Brolin, Hellerstrom & Hellman* 1961) Other characteristics of the islet tissue in the obese-hyperglycaemic animals are a diminished proportion of A-cells (*Gepts, Christophe, & Mayer* 1960, *Hellman* 1961) and signs of increased functional activity of the B cells (*Hellman & Petersson* 1960) In dark field microscopy of fresh pancreatic tissue the islets of Langerhans appear degranulated (*Hellman, Hellerstrom, Larsson & Brolin* 1961), a phenomenon which has been described also after the use of ordinary granule staining methods on Bouin fixed material (*Thiel* 1958, *Petersson & Hellman* 1962) As a result of the raised functional state of the numerous B cells, there is an increased "insulin like" activity in the circulating blood of the obese-hyperglycaemic mice

In considering different factors of importance for the islet changes *in the obese-hyperglycaemic mice attention has been paid to their food* intake It is well known that the caloric surplus in these animals is due partially to their hyperphagia (*Mayer* 1960) The appearance of the islet tissue in the obese-hyperglycaemic mice was found to be greatly influenced by the amount of food consumed, but quantitative analysis revealed that some changes were still present in diet restricted animals maintained for a long time within a normal body weight range (*Petersson & Hellman* 1962) *In the present work the islets of Langerhans* were studied in mice with obesity induced by goldthioglucose This substance is reported to provoke a "regulatory" obesity, probably by hypothalamic lesions (*Mayer* 1960, *Swartz, Christian & Andrews* 1960) The use of naturally non-obese adult mice belonging to the American strain with the recessive trait for the obese-hyperglycaemic syndrome made it possible to get further information on how the increased caloric *intake of the AO-mice affects the islets of Langerhans*

In accordance with our previous observations (*Petersson & Hellman* 1962) a close relationship was noted between the total amount of food ingested and the appearance of the islets The increase of the total islet volume was moderate in comparison with the very marked islet hyperplasia seen *in the obese-hyperglycaemic syndrome (cf Gepts, Christophe & Mayer* 1960, *Hellman, Brolin, Hellerstrom & Hellman* 1961) There are also other observations supporting the view that the hyperphagia is of minor importance for the islet changes in the obese hyperglycaemic syndrome The percentile frequency of silver-positive islet cells is only about half as great in the obese-hyperglycaemic mice

as compared to AN mice of the same age (*Hellman* 1961) In the present work, however, the percentage of these silver positive cells was within normal range also in the mice with obesity induced by goldthioglucose The biological significance of these observations is difficult to interpret since the function of the silver positive A_1 cells is unknown, and the non blackened islet cells include both the insulin secreting B cells and the silver-negative A_2 cells (*Hellman & Hellerstrom* 1961, *Hellerstrom & Hellman* 1961) It is worthy of note also, that the marked reduction of aldehydefuchsin positive material, as found in the B cells of the obese hyperglycaemic mice (*Thiel* 1958, *Petersson & Hellman* 1962), was not observed in the goldthioglucose obese mice

In previous investigations a lower total islet volume and a higher proportion of silver positive cells were noted in lean mice of the strain used here (*Hellman, Brolin Hellerström & Hellman* 1961, *Hellman* 1961, *Petersson & Hellman* 1962) Since these values refer to animals only about 3–5 months old, it is quite possible that large age differences exist with regard to the amount and cellular composition of the pancreatic islet tissue For example pronounced age changes have been demonstrated in the rat islets (*Hellman* 1959)

SUMMARY

The reactions of the pancreatic islet tissue were studied in "regulatory obesity induced by injections of goldthioglucose in naturally lean adult mice from the American strain with the recessive trait for obesity and hyperglycaemia In these obese mice there was an increase of more than 100 per cent in the total islet volume but no significant changes in the percentile frequency of silver positive A cells The results support our previous hypothesis that hyperphagia is a factor of minor importance for the marked changes in the pancreatic islet tissue associated with the obese hyperglycaemic syndrome

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HISTOCHEMICAL STUDIES ON TISSUE CULTURES IN SARCOIDOSIS

By

Anna Lisa Obel, Holger Lundbeck and Sven Löfgren

Received 8.ii.62

The morphological picture of plasma clot cultures unstained as well as stained with *Giemsa* of sarcoidotic lymph nodes and skin lesions was described in a previous paper (*Lundbeck, Löfgren & Nordenstam* 1959). The cultures contained foci within which the fibroblasts were gradually rounded off and contained faintly eosinophilic inclusions which with phase contrast microscopy gave a strong red colour. The morphological picture in such cultures has been described previously (2-4). The present paper deals with the histochemical changes.

MATERIALS AND METHODS

Lymph nodes taken by *Daniels* operation from sarcoidosis patients were used for direct tissue cultivation. The tissue culture technique has been described elsewhere (*Lundbeck, Löfgren & Nordenstam* 1959).

RESULTS

In the fibroblast carpet which after a few days surrounded the lymph node fragments small areas developed where the fibroblasts were rounded off and had lost their processes. The cytoplasm became strongly basophilic (Fig. 1). Since the basophilia disappeared after treatment with ribonuclease it was due to large amounts of ribosenucleotides. The content of ribonucleotides was sometimes so great that the nucleus was covered and fully obscured. All stages of development from typical fibroblasts to cells of irregular shape and to rounded cells about the size of large lymphocytes were observed (Fig. 2). In *Feulgen* stained slides the cytoplasm was unstained and accordingly contained no ribodesosenucleotides (Fig. 3).

In the majority of cells within these particular formations uncoloured or faintly eosinophilic cytoplasmic inclusions could be demonstrated. The inclusions were like small rounded granules or large homogeneous masses. Often they could not be detected by staining with

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Giemsa since they were covered by the ribosenucleotides also present (Fig 1 and 2) Through phase-contrast microscopy of the *Giemsa* stained slides these inclusions became easily visible with their bright red colour With periodic adic and the *Schiff* reagent these structures were strongly PAS positive and clearly demarcated against the PAS-negative cytoplasm (Fig 4 and 5)

Substances mainly responsible for positive PAS reactions contain glycoles in 1 2 position PAS staining shows the presence of glycogen neutral mucopolysaccharides muco and glycoprotein glycolipids unsaturated lipids and phospholipides (*Pearse* 1960) In attempts to analyze the PAS positive substance some histochemical and enzymatic reactions have been carried out

After treatment with diastase the PAS positive substance was still PAS positive Glycogen can thus be excluded

The PAS positive substance did not bind methylene blue below pH 4 and did not give any metachromasia by staining with Azure A or Toluidin blue These reactions are typical for neutral mucopolysaccharides and muco and glycoproteins and indicate that the substance does not contain acid mucopolysaccharides Attempts to stain the substance with Alcian blue and according to *Hale* methods which are comparatively specific for acid mucopolysaccharides gave similarly negative results Even digestion with hyaluronidase did not influence the result with the PAS staining Evidently thus the substance did not contain hyaluronic acid

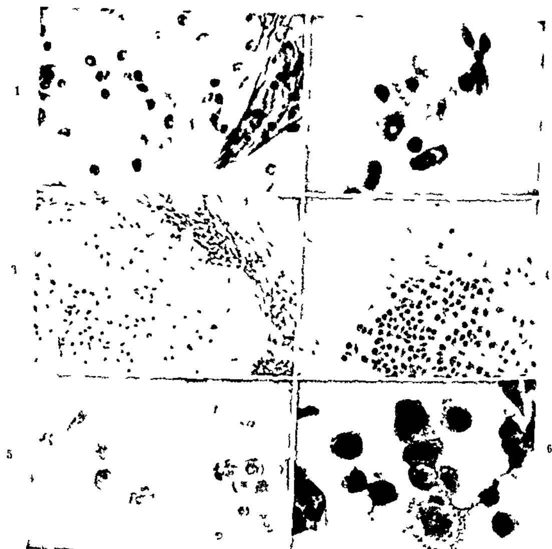
With Sudan Black B a slightly positive diffuse reaction in the cytoplasm was obtained However the areas where the PAS positive substance was found were not stained stronger than the other parts of the cytoplasm It can therefore be excluded that the substance consists of glyco or phospholipids or unsaturated lipids

By treating the slides with pyridine according to *Kramer & Windrum* (1954) sulphate groups were introduced into the PAS positive substance Metachromasia was hereby obtained when staining with Azure A This reaction indicates that the substance is a polysaccharide which can be esterized (Fig 6)

DISCUSSION

The histochemical investigations which have been performed show that the PAS positive substance is *probably a neutral* mucopolysaccharide or muco or glycoprotein These substances cannot yet be distinguished by histochemical methods

As the neutral mucoposaccharides have been found only in gastric mucin it seems more probable that the substance should belong to the group of muco and glycoproteins which occurs in fractions of serum albumin serum globulin and collagen The strong PAS reaction speaks in favour of a mucoprotein



Figs 1–6

Fig 1 Lymph node culture maintained for 6 days Part of a herd in the fibroblast carpet where most of the cells are rounded off and have lost their processes The herd is surrounded by densed streaks of fibroblasts The cells in the herd contain large amount of ribonucleotides which renders them strongly basophilic In some of them a lighter spot is visible in the cytoplasm (the PAS positive subtsance) Giemsa × 300

Fig 2 Detail of the herd in the preseding figure The cells contain various degrees of *ribonucleotides most in the smallest cells The PAS positive substance* [illegible] × 500

Fig 3 [illegible] ribo [illegible] l and [illegible] posite

Fig 4 Lymph node culture maintained for 9 days The rounded cells in [illegible] well demarcated herd contain large amounts of PAS positive substance in the cytoplasm PAS × 150

Fig 5 [illegible] mall [illegible] The [illegible] 500

Fig 6 [illegible] ldes [illegible] the PAS positive substance in the cells in the herds becomes [illegible] atic and stains red violet with Azure A Sulphation metachromasia method of *Kramer* and *Windrum* × 500

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It is of theoretical interest that the fibroblasts in tissue culture in their cytoplasm can produce a PAS-positive substance, possibly a mucoprotein. It has been extensively discussed if PAS-positive substances of the same nature which under pathological circumstances occur in the tissues are *derived from the blood or produced in situ*. Our observation supports the hypothesis (*Teilum* 1956), that such substances can be produced by the cells. On the basis of the observations described studies of the mucopolysaccharides in the blood of sarcoidotic patients was performed by *Norberg*. She observed a slight to moderate increase in glycoprotein in all acute and progressive cases. In the most advanced cases there was a great increase in glycoprotein.

In the sarcoidotic lymph nodes, such deposits are a typical and common occurrence. *Teilum* (1948) who considered the deposits to be paraamyloid, was of the opinion, that they were caused by an immunity reaction.

As stated elsewhere (*Obel, Lundbeck & Lofgren* 1961) the changes described have also been found in tissue cultures of lymph nodes with non-specific lesions. They appeared, however, more regularly and abundantly in cultures from sarcoidotic lymph nodes.

SUMMARY

Lymph nodes obtained by *Daniels'* operation from sarcoidosis patients were used for direct tissue cultivation. In the fibroblast-carpet which after a few days surrounded the lymph node fragments small areas developed where the fibroblasts were rounded off and had lost their processes. The cytoplasm was strongly basophilic due to large amounts of ribonucleotides. The cytoplasm contained uncoloured or faintly eosinophilic inclusions which appeared like small granules or large homogeneous masses. Under phase-contrast microscopy, these inclusions stained bright red with *Giemsa* and with periodic acid and the Schiff reagent they were strongly PAS-positive. Histochemical investigations showed that the substance probably is a neutral mucopolysaccharide or muco- or glycoprotein.

This change in the fibroblasts occurs invariably in lymp nodes from sarcoidosis patients but they have been found in lymph nodes removed from patients with diseases other than sarcoidosis. The change is consequently not specific for sarcoidosis.

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In the sarcoidotic lymph nodes, such deposits are a typical and common occurrence. *Teilum* (1948) who considered the deposits to be paraamyloid, was of the opinion, that they were caused by an immunity reaction.

As stated elsewhere (*Obel, Lundbeck & Lofgren* 1961) the changes described have also been found in tissue cultures of lymph nodes with non-specific lesions. They appeared, however, more regularly and abundantly in cultures from sarcoidotic lymph nodes.

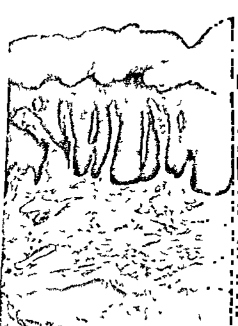
SUMMARY

Lymph nodes obtained by *Daniels'* operation from sarcoidosis patients were used for direct tissue cultivation. In the fibroblast-carpet which after a few days surrounded the lymph node fragments small areas developed where the fibroblasts were rounded off and had lost their processes. The cytoplasm was strongly basophilic due to large amounts of ribonucleotides. The cytoplasm contained uncoloured or faintly eosinophilic inclusions which appeared like small granules or large homogeneous masses. Under phase contrast microscopy, these inclusions stained bright red with *Giemsa* and with periodic acid and the Schiff reagent they were strongly PAS-positive. Histochemical investigations showed that the substance probably is a neutral mucopolysaccharide or muco- or glycoprotein.

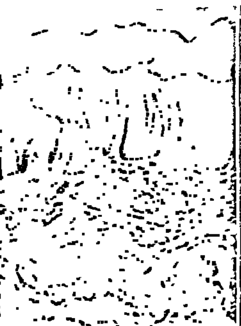
This change in the fibroblasts occurs invariably in lymp nodes from sarcoidosis patients but they have been found in lymph nodes removed from patients with diseases other than sarcoidosis. The change is consequently not specific for sarcoidosis.

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Norberg, Renee: Personal communication



A Photomicrograph of haemalum eosin stained section from area influenced by snuff The homogeneous band is acidophilic × 52



B Photomicrograph of same specimen as in A In van Gieson's stain the homogeneous tissue is yellow × 52



C. Photomicrograph of same specimen as in A and B stained with periodic acid Schiff reagent after preceding diastase incubation The homogeneous tissue is strongly PAS positive and diastase-resistant × 52

FROM THE DENTAL DEPARTMENT, THE UNIVERSITY HOSPITAL AND THE DEPARTMENT OF ORAL PATHOLOGY, THE ROYAL DENTAL COLLEGE COPENHAGEN AND THE DEPARTMENT OF DERMATOLOGY, THE FINSEN INSTITUTE

STUDIES IN ORAL LEUKOPLAKIAS

1 *The Influence of Snuff Upon the Connective Tissue of the Oral Mucosa Preliminary Report*

By

J J PINDBORG and H E POULSEN

Received 11 II 62

Most of the reports describing the effects of snuff upon the oral mucosa are merely clinical The carcinogenic nature of snuff has been questioned, although it is admitted that long-standing snuff dipping may cause leukoplakia (2) It is known that snuff gives rise to hyperplasia of the oral epithelium (1), but the reaction of the underlying connective tissue has not been subject to detailed investigation

Four out of seven male patients using snuff exhibited a peculiar change in the connective tissue in the affected area They had all used snuff (of the brand called Gothenburg-snuff) for periods varying from 20 to 30 years, and placed the quid of snuff in the alveolar sulcus corresponding to the lower incisors Here, the mucous membrane was slightly whitish and had a delicately folded appearance

In local anesthesia a biopsy was taken from the affected area The tissue was fixed in formaline and embedded in paraffine The sections were stained with haemalum-eosin, van Gieson's connective tissue stain, and periodic acid Schiff reagent with and without diastase pretreatment

The histologic investigation reveals a thickening of the squamous epithelium and areas of homogeneous tissue at the transition between lamina propria and tela submucosa The homogeneous tissue is seen either as a 150 μ wide band parallel to the surface of the epithelium or as small areas in connection with glandular tissue The homogeneous tissue stains acidophilic with haemalum-eosin (Fig A) and yellow with van Gieson's stain (Fig B), thus indicating the tissue being of a non-collagenous nature Staining with periodic acid Schiff reagent, with and without diastase pretreatment, shows the changed tissue to be strong PAS positive and resistant to diastase treatment (Fig C)

From these preliminary observations it can be concluded that long-

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FROM THE VIRUS DEPARTMENT NATIONAL INSTITUTE OF PUBLIC HEALTH OSLO NORWAY (HEAD O LAHELLE MD)

THE HETEROLOGOUS, ANAMNESTIC HI-ANTIBODY INCREASE RESULTING FROM CROSS INFECTION WITH INFLUENZA A_2 AND DUTCH '56 A_1-STRAINS

By

ARILD HARBOE

Received 25 1 62

This paper gives an account of various experiments concerned with the formation and specificity of the haemagglutination inhibiting (HI) antibodies produced anamnestically when an infection with influenza virus of the Dutch '56 A_1-type takes place before or after an infection with influenza A_2 virus (3, 4)

MATERIALS AND METHODS

Haemagglutination inhibition (HI) test It was proceeded as described in a previous paper (9) The sera were pretreated with cholera filtrate Diluted infected chick allantoic fluids were employed as test antigens

[illegible] cer [illegible] tre [illegible] n the [illegible] spun down at 25 000 [illegible] residual [illegible] virus was added to 4 or 9 volumes of cholera treated serum As a rule 3 absorption cycles were performed permanent titres were then obtained

The ferret sera were drawn from animals infected by intranasal instillation of one ml of virus bearing chick allantoic fluid

All A_1 strains except FM 1 belonged to the Dutch '56 subtype For short 'A_1' therefore is used synonymously with 'Dutch '56 A_1'

RESULTS

1 *Cross infection with A_2 followed by A_1/Denmark/2 57* In a previous study (3) the two Dutch '56 A_1 strains which were tested (A_1/Netherlands 36 56 and A_1/Denver/1/57), provoked a 4 fold and greater anamnestic A_2 titre increase in cross infected ferrets In the present investigation a third strain of the Dutch '56 type was tried, A_1/Denmark/2 57 (9)

It was found that when a ferret was infected with this A_1-strain 15

The author is indebted to Miss G Thurmann Wang and to Mr A Bye Hansen for skilled technical assistance

standing use of snuff dipping may cause a deposit of a PAS-positive diastase-resistant substance in the oral mucosa Further studies are being carried out attempting an identification of this compound

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in a ferret which was dripped intranasally with formalin-killed A_2-virus first and with the live A_1-strain 5 months later

3 *Antibody cross-absorptions in sera from ferrets cross-infected with A_2 followed by Dutch '56 A_1* Previously the author had found (4), that A_2 antibodies produced anamnestically in ferrets cross-infected with A_2 followed by Dutch '56 A_1 could be absorbed by the A_1-virus On the other hand A_2 virus failed to absorb A_1-antibodies in these sera The results of these cross-absorption tests were different from those of *Jensen & al* (10) They studied sera from ferrets cross-infected with different pre-Asian A strains, and found that the virus which had produced the first infection, completely absorbed the antibodies inhibiting the subsequent strains while the latter absorbed only a fraction of the antibodies to the preceding However, contrary to A_1 and A_2, the strains studied by *Jensen & al* showed direct cross-inhibition

In the present investigation cross-absorption tests were performed with sera from ferrets 143 and 149 mentioned above

The A_2–A_1 ferret 143 Table 1 shows that absorption with A_2-virus reduced the A_1-titre of the 10 day-sample after cross-infection, but not of the later samples When the experiment was repeated with the 10 and the 15 day-samples, a 2-fold A_1 titre reduction was recorded with the former, and no reduction with the latter sample In a repeated experiment with the 10 and the 63 day-samples a 4 fold A_1-titre reduction was recorded with the former, and a 2 fold with the latter sample

Also the A_2-titres after absorption with A_1-virus are shown in Table 1 It is seen that some of the antibodies responsible for the A_2-titre increase were not absorbed

The A_2–A_1 ferret 149 Treatment with A_2 virus resulted in less than a 2 fold A_1-titre reduction in the 10 and the 75 day-samples after the cross infection

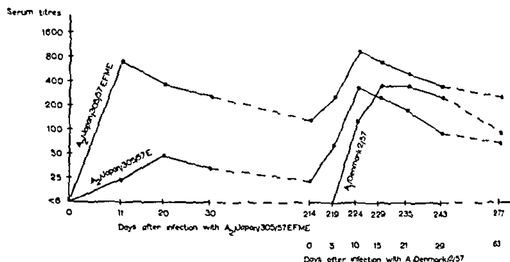
Treatment with A_1 virus reduced heavily the A_2-titres in the 10 days and later samples after the cross infection, as shown in Table 2

In another experiment the 20 day-sample after cross infection underwent 4 absorption cycles with different amounts of A_1 virus The resulting A_1 and A_2-titres are shown in Table 3 It is seen that there was no distinct A_2-titre reduction until the A_1-inhibition was abolished

4 *Antibody cross-absorptions in serum from a ferret cross-infected with Dutch '56 A_1 followed by A_2* The animal had received A_1/Netherlands/36/56 and 4 months later A_2/Singapore/1/57, and presented a great anamnestic A_1 titre rise

When the 10 day sample after cross infection was absorbed with A_1 virus the A_2 titres were reduced from 190 untreated, to 30 after one cycle, and with no further reduction after two and three cycles Thus there was a substantial, though not complete absorption of the A_2-antibodies

When the same sample was treated with A_2-virus and tested simultaneously with untreated samples drawn at other intervals, the follow



Haemagglutination inhibiting antibodies in the cross infected ferret 143
EFME is short for egg ferret-mouse egg line, E for egg line

months after infection with A_2/Singapore/1/57, a 4-fold increase of the A_2-antibody titre took place. A great, anamnestic A_2-titre rise was also presented by an animal, ferret No. 143 (see section 3 below), which was cross-infected with A_2/Japan/305/57 EFME (egg-ferret-mouse-egg line) followed 7 months later by this A_1-strain. The antibody development in ferret 143 is shown by the diagram and Table 1. The titres presented in the diagram were slightly different from those of the table, because they had not been obtained in the same titration. When A_1/Netherlands/36/56 and A_1/Denmark/2/57 were compared as antigens in simultaneous HI-tests, almost identical results were obtained.

2. *A_2-immunization without infection, followed by infection with a Dutch '56 A_1-strain.* Because the ferrets described above and earlier (3) had been *infected* with the two strains, the question arose as to whether the anamnestic antibody increase might be due to a revival of an old infection by a new one.

In order to throw light on this problem ferrets were immunized with A_2-virus under conditions which were not expected to give an infection.

6 ferrets received the A_2-virus subcutaneously. Iodine was painted on the site of the injection to discourage the animals from licking the place and thus become infected (2). 4–16 months later they were infected with A_1/Netherlands/36/56 by intranasal instillation. 3 of the animals responded to the A_1-strain with a 4-fold or greater A_2-titre rise. In the other 3 animals no or only a small A_2-titre change was recorded. One of the former 3 was ferret No. 149, see section 3 below, which had received a subcutaneous injection of one ml of A_2/Norway/9/57, haemagglutinating titre 8,000, and 4 months later had been dripped with the A_1-strain. The anamnestic A_2-titre rise of this animal was very pronounced (see Table 2).

A great anamnestic A_2-titre rise (from < 12 to 192) was also observed

in a ferret which was dripped intranasally with formalin killed A_2-virus first and with the live A_1 strain 5 months later

3 *Antibody cross absorptions in sera from ferrets cross infected with A_2 followed by Dutch 56 A_1* Previously the author had found (4), that A_2 antibodies produced anamnestically in ferrets cross infected with A_2 followed by Dutch 56 A_1 could be absorbed by the A_1 virus On the other hand A_2-virus failed to absorb A_1 antibodies in these sera The results of these cross absorption tests were different from those of *Jensen & al* (10) They studied sera from ferrets cross infected with different pre Asian A strains and found that the virus which had produced the first infection completely absorbed the antibodies inhibiting the subsequent strains while the latter absorbed only a fraction of the antibodies to the preceding However contrary to A_1 and A_2 the strains studied by *Jensen & al* showed direct cross inhibition

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When the 10 day sample after cross infection was absorbed with A_1 virus the A_2-titres were reduced from 190 untreated to 30 after one cycle and with no further reduction after two and three cycles Thus there was a substantial though not complete absorption of the A_2-antibodies

When the same sample was treated with A_2-virus and tested simultaneously with untreated samples drawn at other intervals the follow

TABLE 1

Haemagglutination Inhibition Test on Absorbed Sera from Ferret 143 which Had Been Infected with A_2/Japan/305/57 FIMI Followed 7 Months later by A_1/Denmark/2/57 and Finally Given A_1/Netherlands/36/56 2 Months Afterwards. The Sera Were Tested with A_1/Netherlands/36/56 and A_2/Japan/305/57 FIMI

Days after inoculation with A_2-virus	Days after inoculation with Dutch 56 A_1 virus	Dutch 56 A_1 antibody titres after absorption with A_2-virus: Number of absorption cyles				A_2-antibody titres after absorption with Dutch 56 A_1 virus: Number of absorption cyles			
		0	1	2	3	0	1	2	3
0						<24	<30		
11						1152	960		
20						576	480		
30						288	240		
214	0	<6				144 (108)	120 (113)	(113)	(105)
219	5	9				384 (216)	240 (180)	300 (197)	281 (211)
224	10	288	90	75	94	1152 (864)	480 (360)	600 (450)	563 (422)
229	15	384	360	300	375	1152 (864)	480 (450)	600 (450)	656 (422)
235	21	288	249	225	234	576 (432)	300 (225)	300 (225)	375 (211)
243	29	288	249	225	234	384 (432)	300 (225)	300 (281)	328 (211)
277	63	96	90	94	117	384 (288)	240 (180)	263 (169)	281 (176)
287	Reinoculated with A_1 virus 73	3072	1920	1800	1500	768 (576)	240 (180)	263 (169)	281 (176)

In brackets the results of a repeated experiment

TABLE 2

Haemagglutination Inhibition Test on Sera from Ferret 149, which Had Received a Subcutaneous Injection of A_2/Norway/9/57 and 4 Months later Had Been Intranasally Inoculated with A_1 Netherlands/36/56. Sera Were Absorbed with the A_1 Strain and Were Tested with A_2/Japan/305/57 FIMF and the A_1-Strain

Days after injection of A_2 virus	Days after inoculation with Dutch 56 A_1 virus	Number of absorption cycles with the Dutch 56 A_1 virus												
		0		1		2	3	0		1		2	3	4
		A_2-antibody titres	Dutch 56 A_1 antibody titres	A_2-antibody titres	Dutch 56 A_1-antibody titres	A_2 antibody titres	A_2-antibody titres	A_2-antibody titres	Dutch 56 A_1 antibody titres	A_2-antibody titres	Dutch 56 A_1 antibody titres	A_2 antibody titres	A_2 antibody titres	A_2 antibody titres
14		48	<24	47	<24	44	49							
127	0							18	<18	13		15	16	17
132	5	24	<6	24	<6	22	25	24	<36	23		22	24	23
137	10	288	576	53	<24	30	<33							
147	20	768	1125	160	<24	44	<33							
192	65	192	192	27	<24	<30	<33	288	288	40	<10	15	16	17
		First absorption experiment						Second absorption experiment						

TABLE 1

Haemagglutination Inhibition Test on Absorbed Sera from Ferret 143 which Had Been Infected with A_2/Japan/305/57 EFME Followed 7 Months later by A_1/Denmark/2/57 and Finally Given A_1/Netherlands/36/56 2 Months Afterwards. The Sera Were Tested with A_1/Netherlands/36/56 and A_2/Japan/305/57 EFME

Days after inoculation with A_2-virus	Days after inoculation with Dutch 56 A_1 virus	Dutch 56 A_1 antibody titres after absorption with A_2-virus. Number of absorption cyles				A_2-antibody titres after absorption with Dutch 56 A_1 virus. Number of absorption cyles			
		0	1	2	3	0	1	2	3
0						<24	<30		
11						1152	960		
20						576	480		
30						288	240		
214	0	<6				144 (108)	120 (113)	(113)	(105)
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277	63	96	90	94	117	384 (288)	240 (180)	263 (169)	281 (176)
	Reinoculated with A_1 virus								
287	73	3072	1920	1800	1500	768 (576)	240 (180)	263 (169)	281 (176)

In brackets the results of a repeated experiment

Attempts at virus isolation from these patients were not performed However, the infection took place at a time when A_2-virus was known to be about while the other human A-strains seemed to have disappeared, so it was believed that A_2 had been the causative virus this time, and that the titre rise of antibodies to the other strains therefore was anamnestic

The patient H J When the 25-day-serum from this patient underwent a single absorption cycle with the Dutch '56 A_1-virus, the A_2-titre fell from 48 to 8 The serum was in short supply, therefore only one absorption cycle was carried out —In a control test a serum drawn in October 1957 from a 1½ year old, A_2-convalescent child without Dutch '56 A_1-antibodies, was absorbed with the same A_1-preparation as H.J. In this case no A_2-titre reduction was noted

When the 25 day-serum from H J underwent 2 absorption cycles with A_2-virus, the following titres were recorded *unabsorbed, after one absorption, after two absorptions* Against swine influenza virus: 192, 15, 15 Against PR 8 63, < 10, < 10 Against FM 1 · 54, 15; 15 Against A_1Netherlands 36 56 · 48, 15, 15 It is seen that a distinct titre reduction to these strains took place

The patient H S The 23-day-sample from this patient underwent 3 absorption cycles with Dutch '56 A_1-virus of the batch which was employed for the A_1–A_2 ferret above The following A_2-titres were recorded unabsorbed 1000, after one absorption 135, after two and three no further reduction In an additional test it was found, that when this serum and sera from 7 A_2-convalescent children without Dutch '56 A_1-antibodies were absorbed once with the A_1-preparation, an 8-fold reduction of the A_2-titre took place in the serum from H S, while none of the childrens' sera showed any reduction

The failure to reduce the A_2-titres in the childrens' sera proved that the A_2-titre reduction obtained in serum from H I and H S could not be ascribed to any contaminating A_2-virus in the A_1-preparations

When the 23-day-serum from H S underwent 3 absorption cycles with the A_2 preparation used for H.J, no titre reduction was recorded against swine influenza virus, PR 8 or FM 1 However, against A_1/Netherlands 36 56 a pronounced reduction was observed Unabsorbed 576, after one absorption < 6

These findings are in good agreement with the observation (Table 4) that in H J an anamnestic titre rise to all 4 pre-Asian test viruses took place, while in H S to A_1Netherlands 36 56 only

DISCUSSION

Also in this investigation it was found that cross-infection of previously A_2-infected ferrets with Dutch '56 A_1-strains can provoke a great anamnestic antibody increase to A... ...

ing A1-titres were recorded: *On the day of the A1-infection* < 12, fourteen days afterwards 3,000; on the day of the A2-infection 300, ten days afterwards 3,000; the latter treated with A2-virus (3 absorption cycles) 600

TABLE 3

Haemagglutination Inhibition Test on Absorbed Serum from A2-A1 Ferret 149 The Sample Had Been Drawn 20 Days after the Inoculation with A1/Netherlands/36/56 Test Antigens Were the A1-Strain and A2/Japan/305/57 FFME

Haemagglutinating titres of the absorbing virus A1/Netherlands/36/56	Number of absorption cycles									
	0		1		2		3		4	
	A2-antibody titres	Dutch 56 A1-antibody titres	A2-antibody titres	Dutch 56 A1-antibody titres	A2-antibody titres	Dutch 56 A1-antibody titres	A2-antibody titres	Dutch 56 A1-antibody titres	A2-antibody titres	Dutch 56 A1-antibody titres
1 000			360	480	375	600	375	375	352	352
	384	768								
3 000			360	480	375	300	375	23	352	29
10 000			360	60	300	<19	187	<23	117	<29
30 000			240	15	113	<19	35	<23	<29	<29

To 4 volumes of cholera treated serum was added 1 volume of the 4 dilutions of A1-virus

5 *Antibody cross-absorptions in human sera* H J, born in 1894, and H S, born in 1878, were admitted to Ullevål Hospital in February and March, 1960 respectively, because of influenza with pneumonia A titre rise in the complement fixation test with soluble A-antigen was found by *Dr Ulstrup*, who kindly put the serum samples at the author's disposal Tests with the sera from H S have been reported previously (5) The sera from these patients were chosen for antibody absorption studies because of an increase of pre-Asian as well as A2-antibodies, see Table 4

TABLE 4

Heterologous HI-Antibody Titre Rise in Sera from two Influenza A2 Infected Persons

Patient	Days after the fever began	Antibody titres in the haemagglutination inhibition test against					Complement fixing titres against soluble A-antigen
		Swine influenza virus	PR 8	FM 1	A1 Netherlands 36/56	A2 Japan 305 FFME	
H J	5	9	<9	n t	n t	<6	<8
	13	27	<9	6	<12	<6	256
	25	144	36	18	24	48	n t
H S	5	72	18	72	9	12	<5
	13	72	18	72	576	384	80
	23	72	18	72	576	576	120
	32	72	18	72	576	576	120

It has been shown that some of the anamnestically produced antibodies apparently can not be absorbed by the heterologous antigen while others are absorbed. Perhaps the former are produced by *i*, the latter by *ii*.

It was found previously that when the two antigens are very different from each other, as B Lee and A are considered to be the anamnestically produced antibodies are not cross absorbed (4 6). Accordingly *i* would be responsible for these antibodies. However it has not yet been shown that there is a sharp distinction between antibodies which are absorbed and those which are not. For all that is known so far an animal may possess antibodies with a practically continuous range of reaction coefficients with the two antigens.

One may finally speculate about the purpose of a great anamnestic increase of antibody against an antigen which is so unlike the provoking antigen that there is little direct cross immunity between them. Is the increase meant to be a defense mechanism against a revival of an old infection by a distantly related new one (6)?

SUMMARY

The haemagglutination inhibition (HI) test was employed.—By A_1 is meant the sub type Dutch 56 A_1.

1 The influenza virus strain A_1 Denmark/2 57 provoked in cross infected ferrets a 4 fold and greater anamnestic increase of antibody titre to influenza A_2-virus. This strain is the third one of the Dutch 56 A_1 type examined by this method and all 3 were seen to provoke a great anamnestic increase.

2 Also when the A_2 virus had been killed with formalin or had been injected subcutaneously in order to avoid infection the anamnestic A_2 antibody increase was produced by the A_1 infection.

3 Antibody cross absorption tests were performed with sera from two ferrets which had received A_2-virus first and A_1 virus afterwards. Sera were drawn at different intervals after the inoculation with A_1 virus.

In one of the animals absorptions with A_2-virus distinctly reduced the A_1 antibody titre of the sample drawn 10 days after the cross infection but not the titres of the 15 days and later samples. In the other animal absorptions with A_2 virus did not reduce the A_1 antibody titre in any of the samples.

Absorption tests with the A_1 virus indicated that the cross infection with this virus had produced A_2 antibodies with different affinities to the A_1 virus.

Absorptions with different amounts of A_1 virus gave no distinct A_2-titre reduction until the A_1 inhibition was abolished.

4 Antibody cross absorption tests were performed with serum from a ferret which after ... followed by A_2-virus presented

showed, that all 3 A_1-strains *provoked* in the previously A_2 infected ferrets the production of antibodies *which inhibited A_2-virus and could* be absorbed by A_1-virus This proved that the *A_2-titre increase following* the inoculation with these *A_1-strains had not been caused by any A-virus material* which might have contaminated the A_1-strains

No cross-reaction has been observed between A_2 and Dutch '56 A_1-*virus in HI-tests* on sera from ferrets convalescent from an infection with only one of these two types This shows that *despite the great* anamnestic titre increase and the double specificity of some of the antibodies formed after cross-infection with either virus, the two types can at most be regarded as distantly related

The diagram of the antibody development *in* the *A_2–A_1* ferret 143 confirms, that after an A_2-infection the peak titre is recorded earlier with A_2-virus of high antibody sensitivity than with A_2-virus of low, when pre-treated sera are tested (7, 5) It is further *observed*, that in the anamnestic response there is no such *difference, neither* are the heights of the two peaks so different *from each other*

The cross-absorption experiments with ferret 143 indicated that the *antibodies* responsible for the inhibition of the A_1-virus were more liable to absorption by A_2-virus 10 days after cross-infection than later (Table 1) Possibly some of the cells which formerly had been producers of A_2-antibodies and therefore were present in great numbers, were the first to produce detectable amounts of A_1-antibodies In the meantime A_2-unexperienced cells were made available for the A_1-antibody production, and after 15 days they have dominated this Another possibility could be that a cell might alter the antibody type (or types?) produced as time proceeds after the A_1-inoculation

Of the anamnestically produced A_2-antibodies in the 10-days and later sera a greater fraction was absorbed by A_1-virus in ferret 149 than in ferret 143 (Tables 3 and 2) However, although in ferret 149 these A_2-antibodies apparently were quite easily absorbed by the A_1-virus, all or at least the greater part of them were unable to compete for the A_1-virus with the antibodies which governed the A_1-inhibition (Table 4)

It was not decided whether the anamnestically produced A_2-antibodies which first appeared in the *A –A_1* ferrets, had a smaller A_1-affinity than the later ones (1)

The great, anamnestic increase of the Dutch '56 *A_1-antibodies in* the patient H S , who did not develop titre increase to swine influenza virus, PR 8 or FM 1, seems to *indicate that the Dutch '56 A_1-type is* more related to the A_2-type than the other strains are

It may be assumed that the anamnestic antibody increase which is provoked by a heterologous antigen, is due to

i a release of the first antigen so that it becomes available to antibody producing cells, and or

ii an influence of the second antigen on cells which formerly produced antibody to the first antigen

It has been shown that some of the anamnestically produced anti bodies apparently can not be absorbed by the heterologous antigen while others are absorbed Perhaps the former are produced by i the latter by ii

It was found previously that when the two antigens are very diffe rent from each other as B/Lee and A_2 are considered to be the anam nestically produced antibodies are not cross absorbed (4 6) Accord ingly i would be responsible for these antibodies However it has not yet been shown that there is a sharp distinction between antibodies which are absorbed and those which are not For all that is known so far an animal may possess antibodies with a practically continuous range of reaction coefficients with the two antigens

One may finally speculate about the purpose of a great anamnestic increase of antibody against an antigen which is so unlike the provoking antigen that there is little direct cross immunity between them Is the increase meant to be a defense mechanism against a revival of an old infection by a distantly related new one (6)?

SUMMARY

The haemagglutination inhibition (HI) test was employed —By A_1 is meant the sub type Dutch 56 A_1

1 The influenza virus strain A_1 Denmark/2 57 provoked in cross infected ferrets a 4 fold and greater anamnestic increase of antibody titre to influenza A_2 virus This strain is the third one of the Dutch 56 A_1 type examined by this method and all 3 were seen to provoke a great anamnestic increase

2 Also when the A_2-virus had been killed with formalin or had been injected subcutaneously in order to avoid infection the anamnestic A_2-antibody increase was produced by the A_1 infection

3 Antibody cross absorption tests were performed with sera from two ferrets which had received A_2 virus first and A_1 virus afterwards Sera were drawn at different intervals after the inoculation with A_1 virus

In one of the animals absorptions with A virus distinctly reduced the A_1 antibody titre of the sample drawn 10 days after the cross infection but *not the* titres of the 15 days and later samples In the other animal absorp*tions with A_2-virus did not reduce the A_1* antibody titre in any of the samples

Absorption tests with the A_1 virus indicated that the cross infection with this virus had produced A antibodies with different affinities to the A_1 virus

Absorptions with different amounts of A_1 virus gave no distinct A_2-titre reduction until the A_1 inhibition was abolished

4 Antibody cross absorption tests were performed with serum from a ferret which after infection with A_1 followed by A_2-virus presented

in anamnestic A_1-titre rise Both viruses reduced the heterologous antibody titre

5 Antibody cross absorption tests were performed also with convalescent sera from two patients who presented heterologous antibody *increase after an A_2-infection*

In a patient with a titre increase to swine influenza virus, PR 8, FM 1 and Dutch '56 A_1 in addition *to the A_2-virus, absorption with the latter* reduced the titres to all these strains The other patient presented a titre increase to Dutch '56 A_1 and A_2-virus, but not to swine virus, PR 8 or FM 1 Absorption with A_2-virus reduced the Dutch '56 A_1 titre, while the titres to the former three strains remained unchanged

In both patients the A_2-antibody titre was reduced when absorption was performed with Dutch '56 A_1-virus

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FROM THE VIRUS DEPARTMENT, NATIONAL INSTITUTE OF PUBLIC HEALTH, OSLO NORWAY (HEAD O. LAHELLE, M.D.)

AN ANAMNESTIC ANTIBODY INCREASE IN FERRETS CROSS-INFECTED WITH INFLUENZA A_2-VIRUS AFTER MYXOVIRUSES NOT BELONGING TO THE A-GROUP

By

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Cross-reactions in the haemagglutination inhibition (HI) test between influenza A_2 and other A-strains have been found previously in cross-infection experiments on ferrets (1, 2). On this occasion it was observed also that a ferret which had been cross-infected with the influenza B-strain Lee followed by A_2/Singapore/1/57, developed a Lee-titre increase after the inoculation with the A_2-strain. This observation was surprising, because no HI-cross-reactions between the influenza A and B groups had been reported in the available literature. It was decided therefore to repeat the cross-infection experiments with the two strains mentioned, and to test other A- and B-strains. A_2-virus was compared also with influenza C and Sendai virus. The results are presented in this paper.

MATERIALS AND METHODS

Viruses and sera. The strains Lee BON (an influenza B strain isolated in Australia in 1943), B/England/9.54, C/Taylor 1233 and Sendai virus (HVJ) were obtained through the World Influenza Centre, London, B/Ann Arbor/1/59 through the International Influenza Center for the Americas, Atlanta. B/Johannesburg/9.58 was received from Dr. *J. H. S. Gear.* Information about the A_2 strains has been given in a previous paper (5).

The sera were obtained from ferrets which had been infected either by intranasal instillation of one ml of virus bearing allantoic fluid from chick embryos, or by contact with intranasally inoculated cagemates. The infe[illegible]

[illegible]

In some exp[illegible]

fluid by ad-[illegible]

The author is indebted to Miss *G. Thurmann Wang* and Mrs. *B. Egelandsdal* for skilled technical assistance.

antigens consisted of diluted allantoic fluid from infected duck embryos or tracheo-bronchial washings from infected mice

Serial 2 fold dilutions were performed and the titres were read by inspection of the patterns of the fowl red cells Distinction has been made between the various degrees of haemagglutination and therefore even quite small differences between serum titres were recorded, for convenience arithmetic interpolation was used When serum titres were compared the different sera had been pretreated and tested by strictly parallel procedures Titre differences less than 2 fold were regarded as negligible unless confirmed in repeated titrations

Complement fixation (CF) test The preparation of soluble antigen and the performance of the test were the same as in a previous investigation (4)

RESULTS

1 *A_2 given before or after different myxoviruses* In these cross infection experiments A₂/Singapore/1/57 was given either before or after the following different influenza B-strains, an influenza C strain or Sendai virus The viruses were dripped intranasally with an interval of 7–8 months

A₂/Singapore/1/57 and A₂/Japan/305/57 were used as antigens in the HI-test, the former strain because it had been used to infect the animals Of the latter strain an egg line (E) of low antibody sensitivity and an egg-ferret-mouse-egg line (EFME) of high antibody sensitivity (8) were employed

When available, duck or mouse grown virus material was employed in the HI-test in addition to the chick grown, because the former two had been found not to be inhibited by antibody to chick material (3)

The results are presented in Tables 1 and 2 In order to save space Table 1 presents the A_2-titres recorded with the Lee-A_2 ferret only, these being typical of the group of animals It is seen (Table 1) that the A_2-titres recorded with virus of low antibody sensitivity were still rising at a time when the A_2-titres recorded with virus of high antibody sensitivity were already on the decline This phenomenon has been observed previously in A_2-infected ferrets which had not been inoculated with other myxoviruses before (5)

The results presented in Table 1 proved reproducible in a repeated titration and suggest that an A_2-virus can provoke an anamnestic antibody increase to widely different myxovirus strains —None of the animals in Table 2 presented as much as a 2-fold titre rise when tested with the duck grown A_2 antigen

It would have been advantageous if more than a single animal had been infected with each of these strains However, this procedure was difficult, and instead the further efforts were concentrated upon one of the strains, Lee, for the practical reason that it had already been adapted to ferret, mouse, and chick embryo

2 *Lee followed by A_2* The ferret in Table 1, which was infected with Lee followed by A_2, produced a 2 fold, anamnestic titre increase in response to the A_2-infection, an increase which was confirmed in repeated titrations A similar increase in a Lee—A₂/Singapore/1/57 ferret

TABLE 1

Haemagglutination Inhibition Test on Sera from Cross Infected Ferrets which Received A_2/Singapore/1/57 7–8 Months after the first Strain

Ferret Infected with	Antibody titres against	Days after 1st infection				Days after 2nd Infection			
		0	9-12	18-21	28-31	0	10-12	20-22	31-33
Lee followed by A_2/Singapore/1/57	Lee chick grown	<12	576	1152	768	48	96	72	48
	Lee duck grown	<9	576	1152	576	36	72	54	36
	Lee mouse grown					36	72	48	
	A_2/Singapore/1/57	<6	<6	<6	<6	<6	96	144	144
	A_2/Japan/305/57 E	<6	<6	<6	<6	<6	48	96	144
	A_2/Japan/305/57 LEM	<12	<12	<12	<12	<12	1152	768	384
BON followed by A_2/Singapore/1/57	BON, chick grown	<9	288	288	126	126	144	126	126
	BON duck grown					48	96	48	
B/England 9/54 followed by A_2/Singapore/1/57	B/England/9/54 chick grown	<6	144	192	144	48	72	72	72
	B/England/9/54 duck grown					36	72	36	
B/Johannesburg 9/58 followed by A_2/Singapore/1/57	B/Johannesburg/9/58 chick grown	<9	1728	1728	576	216	288	288	144
C/Taylor 1233 followed by A_2/Singapore/1/57	C/Taylor 1233 chick grown	<6	384	384	384	96	144	96	96
	C/Taylor 1233 duck grown					192	288	192	
Sendai virus followed by A_2/Singapore/1/57	Sendai virus chick grown	<6	48	72	48	12	24	18	12
	Sendai virus duck grown					24	72	24	

TABLE 2

Haemagglutination Inhibition Test on Sera from Cross Infected Ferrets which Received A_2/Singapore/1/57 7–8 Months before the other Strain

Ferret infected with	Antibody titres against	Days after 1st infection			Days after 2nd infection			
		0	10	19	0	10-14	20-23	31-33
A_2/Singapore/1/57 followed by Lee	A_2/Singapore/1/57 chick grown A_2/Japan/305/57 LEMF duck grown	<6	36	48	12 36	12 36	12 36	12
A_2/Singapore/1/57 followed by BON	A_2/Japan/305/57 LEMF chick grown A_2/Japan/305/57 LEMF duck grown	<12	384	144	12 24	24 24	12 24	12
A_2/Singapore/1/57 followed by B/England/9/54	A_2/Singapore/1/57 chick grown A_2/Japan/305/57 LEMF duck grown	<6	96	96	18 54	18 36	18 36	18
A_2/Singapore/1/57 followed by B/Johannesburg 1/58	A_2/Singapore/1/57 chick grown A_2/Japan/305/57 LEMF duck grown	<6	144	192	24 96	36 96	24 96	24
A_2/Singapore/1/57 followed by C/Taylor 1233	A_2/Singapore/1/57 chick grown A_2/Japan/305/57 LEMF duck grown	<6	24	24	<12 24	<12 24	<12 24	<12
A_2/Singapore/1/57 followed by Sendai virus	A_2/Japan 305/57 LEMF chick grown A_2/Japan/305/57/ LEMF duck grown	<6	24	12	6 24	6 18	6 12	6

had been observed in a previous investigation (1) An analogous observation had been made in a 20 year old man during an A2-epidemic (4)

In order to see whether the anamnestic B antibody rise could be experimentally produced with another A strain than A2Singapore/1 57 ferrets were cross infected with Lee and the strain A Japan 305 57 the FFMF line The chance that both of these A2 viruses were contaminated with influenza B virus appeared small

In the following experiment a ferret No 1 was dripped intranasally with Lee infected allantoic fluid the usual way The following day another ferret No 2 was brought into the same cage and became spontaneously infected as revealed by Lee antibody development (see below) Eight months later both ferrets together with 2 other ferrets *a* and *b* were transferred to a cage housing a ferret *c* which the day before had been dripped with A Japan 305 57 1 FMI All 5 animals subsequently developed A antibodies

The Lee-titres of ferret No 1 were Before the Lee infection < 6 ten and twenty days after 2300 eight months after 192 Ferret No 2 correspondingly gave < 6 96 < 6 Two weeks after the last serum samples had been drawn the animals were exposed to the A2 virus Ten days after this exposure the Lee titre of ferret No 1 was 288 No 2 however presented the Lee titre 144 a remarkable increase as compared with < 6 before the A infection This increase was therefore subjected to a closer study

Speaking against an accidental reinfection of ferret No 2 with a B strain was the failure to detect antibodies to Lee or to a recent B strain B Ann Arbor 1 59 when sera from the cagemates *a* *b* and *c* after the A infection were examined in the HI test It was checked also that ferret No 2 had not developed antibodies to a recent B strain Neither could the increase in this ferret be due to a failure of the pretreatment with cholera filtrate by which to abolish *normal serum* inhibitor activity This was proved by means of an indicator virus preparation obtained by heating Lee infected chick allantoic fluid at 56° C for ½ hour The indicator virus gave 30 times *higher titres* than the original active virus when tested with normal ferret serum which had been heated at 56 C for ½ hour When sera from ferret No 2 before and after the A2-infection were tested after the pretreatment with cholera filtrate the heated Lee gave practically *the same* titre rise as the active Lee — A CF test with soluble B antigen was performed but failed to give a conclusive answer because none of the sera from this ferret showed specific fixation

If it could be demonstrated that antibodies of a double spec[illegible] (anti A Lee molecules) [illegible]
A2-infection it would [illegible]
observed after the A in[illegible] [illegible]duced by this infection
Therefore attempts at heterologous antibody absorptions were per

formed (2) One volume of virus, haemagglutinating titre about 50,000, was added to 4 volumes of serum which had been diluted to 1:6 by the pretreatment with *cholera filtrate* 3 absorption cycles were performed However, despite these intensive virus treatments, the Lee-antibody titre was not distinctly reduced when the serum was treated with A_2/Japan/305/57 E, neither was the A_2-titre reduced by Lee It has to be stressed that the negative outcome of the absorption experiments does not disprove the assumption that the Lee-titre rise observed in ferret No 2 after the A_2-infection is an anamnestic response to the latter

The following experiment was an attempt to provoke a similar increase in other Lee-A_2 ferrets Ferret No 2 had been infected on both occasions by animal contact, and therefore it was tried to get some more ferrets crossinfected by this method However, of 7 ferrets exposed, none caught both infections spontaneously, at least no detectable antibodies against the strain concerned developed, and hence it was decided instead to infect as usual by intranasal instillation of infected chick allantoic fluids

Three ferrets, Nos 3, 4 and 5, were inoculated with Lee and 4-7 months later with A_2/Japan/305/57 ETME In order to examine the influence of antibody against host material on the titres recorded, the animals received one ml of normal chick allantoic fluid intranasally 10 days before the A_2-inoculation The sera were tested with the following preparations of Lee-virus

(1) Diluted, infected allantoic fluid from chick embryos
(2) An indicator virus preparation which was 15 times as sensitive than the active virus to normal ferret serum inhibitor
(3) Diluted, infected allantoic fluid from duck embryos
(4) Tracheo-bronchial washings from infected mouse lungs
(5) Virus eluate, which was employed in the absence and in the presence of normal chick allantoic fluid treated with equal amounts of cholera filtrate and absorbed with fowl red cells (4)

The results of these titrations are shown in Table 3, which also by means of swine influenza virus eluate (4), demonstrates the appearance of antibody to host material

Table 3 shows that in none of the three ferrets the A_2-inoculation provoked a 4-fold or greater titre increase to any of the Lee-antigens However, a small increase followed by a similar decrease was frequently noted, a decrease instead of an increase was never observed Because there was no difference in this respect between the various Lee-antigens, the titre rise could not be explained as due to normal inhibitor activity, or to the antibody produced against host material and revealed by means of the swine virus eluate The latter antibody had been masked obviously by the more hightitred virusspecific Lee-antibodies (4) —The negative result of the instillation of normal allantoic fluid

spoke against the titre rise being a response to the procedures connected with bleeding and inoculation

The absence of contaminating Lee-virus in the A_2-inoculum was checked as follows A_2-infected allantoic fluid from the same batch

TABLE 3

Haemagglutination Inhibition Test on Sera from Ferrets Cross Infected with Lee Followed by A_2/Japan 305 57 FFME 4 7 Months later 10 Days before the Inoculation with A_2 Virus the Animals Received Intranasally 1 ml of Normal Chick Allantoic Fluid—The Lee Titre Rise and Subsequent Fall in the 0 10–20 Days Samples Were Confirmed in Repeated Tests on the Sera from Ferrets 3 and 4

Ferret No	Antibody titres when tested with	Days after 1st infection (Lee)	Days before	days after 2nd infection (A)			
		14-16	10	0	10	20	31
3	Lee chick grown	1536	192	192	288	192	192
	Lee chick grown heated	1536	96	96	144	96	96
	Lee chick grown an eluate	2304	288	288	384	288	192
	Lee chick grown an eluate + normal chick allantoic fluid		288	288	384	192	
	Lee duck grown			168	336	192	
	Lee mouse grown			144	192	144	
	A_2/Japan/305/57 EFME			<6	576	288	192
	Swine influenza virus chick grown an eluate			<9	18	<9	<9
	Swine influenza virus chick grown an eluate + normal chick allantoic fluid			<18	<9	<9	
4	Lee chick grown	1152	288	288	384	288	288
	Lee chick grown heated	1536	384	288	384	288	288
	Lee chick grown an eluate	1536	384	384	576	384	384
	Lee chick grown an eluate + normal chick allantoic fluid		384	384	768	384	
	Lee duck grown			288	384	288	
	Lee mouse grown			192	384	192	
	A_2/Japan,305/57 EFME			<6	768	384	192
	Swine influenza virus chick grown an eluate			<9	18	9	<9
	Swine influenza virus chick grown an eluate + normal chick allantoic fluid			<9	<9	<9	<9
5	Lee chick grown	3072	384	384	384	384	384
	Lee chick grown heated	3072	384	384	384	384	384
	Lee chick grown an eluate	3072	384	384	576	576	576
	Lee chick grown an eluate + normal chick allantoic fluid			768	768		
	Lee duck grown			384	576		
	Lee mouse grown			192	288		
	A_2/Japan/305 57 EFMF			<6	768	384	384
	Swine influenza virus chick grown an eluate			<9	36	14	9
	Swine influenza virus chick grown an eluate + normal chick allantoic fluid			<9	<9	<9	<9

which had been used to infect the three ferrets (Nos 3, 4 and 5), was dripped intranasally into four ferrets which had not been inoculated before The A_2-inoculation did not result in detectable Lee-antibody development in the four animals or in five others which became A_2-infected by contact with the four —Equally negative was an attempt to detect any contaminating myxovirus in this particular batch by inoculating eggs with mixtures of the allantoic fluid and falling dilutions of antiserum against another A_2-strain The only haemagglutinating virus which appeared, proved to be an A_2-strain

3 *Lee-antibody increase of unknown origin* The day after ferrets Nos 3, 4 and 5 (previously infected with Lee, see the foregoing section) had been inoculated with A2/Japan/305/57 EFME, 4 ferrets, Nos 6 7 8 and 9, were transferred to the same cage where the former stayed Also the latter animals had been infected with Lee 5–6 months ago, No 9 by animal contact, the other 3 by intranasal instillation However the contact with the 3 A_2-inoculated ferrets did not result in detectable A_2-antibody development in Nos 6, 7, 8 or 9

A comparison was made between the Lee-titres in sera obtained from the latter 4 ferrets

I 10 days before the A_2-inoculation of their cagemates
II On the day of this inoculation
III 12 days after inoculation

No increase was observed between the samples I and II in any of the 4 ferrets There was, however, an increase not exceeding half a dilution step between II and III in Nos 7, 8 and 9, while No 6 showed no change This increase, though small, was well reproduced in repeated titrations with active and with heated virus Of 20 titre pairs 15 gave an increase, and 5 presented no changes The observed titre increase therefore may reflect an increase of the Lee-anitibody content, which in an unknown way might have been provoked by the contact with the newly A_2-inoculated, previously Lee-infected animals —Subsequent samples showed that the small increase between II and III did not represent the initial stage of a larger increase

4 *A_2 followed by Lee* These crossinfection experiments were performed with 4 animals infected by intranasal instillation, of A2/Japan/305/57 EFME and 5 animals which became A_2-infected by contact with the former

About 5 months after the A_2-infection 4 animals (2 from the former and 2 from the latter group above), group *Inoc* were inoculated intranasally with Lee, the following day the remaining 5, group *Cont*, were transferred to a cage in which group *Inoc* was kept, so that group *Cont* might become Lee-infected by contact with the former However, no Lee-antibodies appeared in the latter group, and it was therefore dripped with this strain, 24 days after group *Inoc*

Only one of the animals, ferret M, responded to the Lee-inoculation

with an A-titre rise. This animal had been A-infected by contact and belonged to group *Inoc*; the titres are shown in Table 4. In the other 8 animals the Lee inoculation did not result in an A-titre rise, not even of half a dilution step.

The Lee inoculation produced not even a 4-fold increase of the antibody titre to soluble A antigen in any of the 9 animals. Chick grown antigen could not be employed for this test, because it fixed complement with the antibody which the ferrets had produced against the chick material in the inoculum, see Table 4. Instead, a soluble A antigen prepared from PR 8 infected mouse lungs was employed.

5. *A-antibody increase of unknown origin.* Ferret N, A-infected by contact and belonging to group *Cont*, which so far had not been dripped with Lee, presented a significant A-titre rise 2 weeks later than its cagemate ferret M above, as shown in Table 4. Simultaneously with this titre rise ferret N developed complement fixing antibodies to soluble A antigen. None of the remaining 4 animals belonging to group *Cont* presented a 2nd titre rise in the HI test with A-antigen or in the CF test with soluble A antigen; not even a 2-fold increase was observed.

DISCUSSION

The anamnestic increase of influenza B and C and of Sendai virus antibody titres which A-infection was seen to provoke was quite small except in a single one of the Lee—A ferrets. Although small, an increase was regularly met with, however, under these experimental conditions. It appears as if the tendency of A-virus to provoke an anamnestic increase of antibody to B, C and Sendai virus was greater than the tendency of these strains to provoke an anamnestic A-antibody increase.

Table 5 is an attempt to range various degrees of antigenic relationship between myxoviruses, based on the HI test.

Different theories may be advanced to explain the second increase of A-antibodies in ferret N (see section 5 and Table 4).

a. The animal has become reinfected with A-virus from a source outside the cage. If so, it probably was an A-strain, because there were no detectable HI antibodies to swine influenza virus, PR 8, FM 1 or A₁ Netherlands 36/56 in the ferret.

b. The old A-infection has flared up spontaneously *in the animal*.

c. The animal has become reinfected by a cagemate, which by the Lee infection has become capable of producing and spreading A-virus.

While *a* is a plausible explanation, *b* and *c* are highly hypothetical as it is unknown whether such phenomena may occur at all. However, there is no direct evidence against *b*. And as regards *c*, a mechanism like this might explain the unexpected Lee titre increase between the serum samples II and III from ferrets 7, 8 and 9 (section 3). Already sensitized to Lee by the old infection the antibody producing system

TABLE 4

The Antibody Development in the Cross Infected Ferrets M and N Described in Sections 4 and 5
Not enough Serum Was Left to Perform a CF Test with all Samples from Ferret N

Ferret M	Days before	Days after the first infection (A2 Japan 305/57 EEMF)								
	2	7	12	27	128	138 Infected with Lee	148	158	168	
HI titres when tested with										
A2/Japan/305/57 EEMF	<6	96	768	576	384	192	768	384	288	
A2/Japan/305/57 E	<9	<9	<9	18	18	18	54	27	27	
Lee	<6	<6	<6	<6	<6	<6	1536	1536	1152	
CF titres when tested with										
Soluble A antigen from infected mouse lungs	<4	4	12	32	24	12	12	12	12	

Ferret N										
	2	7		27	128	139	151	161	162 Infected with Lee	172
HI titres when tested with										
A2/Japan/305/57 EEMF	<6	<6		288	96	96	96	384		192
A2/Japan/305/57 E	<9	<9		9	14	14	14	54		27
Lee	<6	<6		<6	<6	<6	<6	<6		1536
CF titres when tested with										
Soluble A antigen from infected mouse lungs (Titres to normal membranes in brackets)	<3(<3)			4(<2)			<2(<2)	4(<2)		
The test repeated							<1(<1)	4(<1)		8(8)

TABLE 5

Patterns of Cross Reactions in the Haemagglutination Inhibition Test between some more and less Related Myxovirus Strains A_0 Means the Human A Strains Known until the Appearance of the A_1 Strains

Degrees of antigenic relationship

(practically) identical	e g All A_0 or B from a single epidemic wave	No persistent serological difference detectable
++++	WS and PR 8	Antibody molecules reacting with both strains appear already after contact with the first strain (direct cross-inhibition)
+++	A_0 and A_1	Antibody formation following contact with the second strain seems to favour the antibodies directed against the first strain so that these reach high titres while formation of antibodies directed against the second strain is suppressed
++	A_2 and Dutch 56 A_1	Antibody molecules reacting with both strains may appear after contact with the second strain and can then be detected in cross absorption tests
+	A_2 and B C or Sendai	Antibody titre against the first strain is more or less regularly increased after contact with the second strain
unrelated	*Myxoviruses compared with viruses belonging to other groups*	*No influence on the antibody titres of each other is detectable*

of these ferrets might have been able to respond, with a just detectable Lee titre rise to a very slight exposure to Lee virus transmitted from their previously Lee-infected newly A_2 inoculated cagemates It remains to be seen whether these speculations would be substantiated by a direct experimental approach

SUMMARY

In cross infected ferrets influenza A_0 virus could provoke an anamnestic antibody titre increase to different influenza B strains, to influenza C and Sendai virus in the haemagglutination inhibition test Usually this increase was not greater than 2 fold

When A_2 virus was given first and B, C or Sendai virus later, no anamnestic A_2-titre rise was observed except for a 4-fold rise in one of the nine ferrets cross infected with A_2 and the B-strain Lee, the other strains had been given only to one animal each

A table ranging different degrees of antigenic relationship between myxovirus strains is presented

A spontaneous titre rise was observed on two different occasions, and various possibilities were discussed

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GLUCOSE CATABOLISM IN NEISSERIA MENINGITIDIS

2. *Reactions of the Pentose Phosphate Pathway and of the Entner-Doudoroff Route*

By

K. JYSSUM and SIDSEL JYSSUM

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Previous experiments have demonstrated that cell free extracts from Neisseria meningitidis contain the enzyme systems necessary for a dissimilation of glucose through the conventional Embden-Meyerhof route.

Studies of the over-all oxidation of glucose and several glycolytic intermediates showed that meningococci also oxidize 6-PG and R-5-P, while the corresponding non-phosphorylated compounds remain unattacked (*Jyssum, Borchgrevink & Jyssum* 1961).

The subject of the present paper is an investigation into the enzyme catalysed transfers during the catabolism of 6-PG and R-5-P. The studies were started mainly in order to obtain information concerning pathways of "primary" glucose oxidation other than the Embden-Meyerhof route.

MATERIALS AND METHODS

The methodology and experimental manipulations used in this investigation were analogous to those previously described (*Jyssum, Borchgrevink & Jyssum* 1961, *Jyssum & Jyssum* 1962). The following additional analytical procedures or modifications were employed.

Cell free extracts. An extraction after ultrasonic disintegration was used as the regular procedure (*Jyssum & Jyssum* 1962). In some experiments this method was compared with the previous extraction after manual grinding of the cells [illegible] glass powder (*Jyssum, Borch*[illegible]

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and deducted from the quantity of the actual sample Since the colour yielded per μg of pyruvic acid as well as of glyceric aldehyde in terms of μg lactic acid are known, the corrected value of lactic acid in the sample could be obtained by subtracting the values corresponding to pyruvic acid and glyceric aldehyde (*Colowick & Kaplan 1955–1957*)

Analyses by enzymatic methods 6–PG was determined enzymatically with yeast 6–PG dehydrogenase and fructose 6-phosphate with phosphohexose isomerase plus glucose-6-phosphate dehydrogenase GA–3–P was determined as previously described with triosephosphate isomerase plus α-glycerophosphate dehydrogenase Pyruvic acid was determined with lactic dehydrogenase

These analyses were regularly performed in solutions which had been deproteinized with perchloric acid (*Jyssum, Borchgrevink & Jyssum* 1961) The influence of the concentration of P_i which resulted from these procedures was explored and found to be without influence on the analytical results

Some inhibitors generally used in the present experimental series *i e* iodacetate arsenite, cyanide and fluoride did not interfere with the results of these analytical procedures

Chemicals In addition to the reagents previously mentioned the following enzymes were purchased from the Sigma Chemical Co 6 PG dehydrogenase G 6 P dehydrogenase and phosphohexose isomerase Sodium 6 PG and sodium R 5 P were also the products of the same firm

RESULTS

6-phosphogluconate dehydrogenase Meningococcal extracts can synthetize 6–PG from G–6 P by a reaction corresponding to the G–6 P dehydrogenase From oxidation experiments with whole cells it is evident also that 6–PG is further metabolized by this microbe (*Jyssum, Borchgrevink & Jyssum* 1961) The existence of a pentose phosphate pathway was accordingly explored

In various meningococcal extracts activities corresponding to a 6 PG dehydrogenase were first searched for and measured Meningococcal extracts were capable of reducing TPN with 6–PG as the substrate, while DPN could not be reduced in similar technique The activities which could be measured in crude extracts, however, depended very much upon the extraction procedure employed When the cells were ground with glass powder the activity corresponding to this enzyme was always very low A much better activity was obtained when the cells were sonically disintegrated

A comparison of the two extraction procedures showed that more nitrogenous material was released from the cell debris to the supernatant fraction when the cells had been treated with ultrasonic waves than when ground with glass under otherwise identical conditions

The activities of the G–6–P and 6 PG dehydrogenases were measured in several extracts When $\Delta A_{340}\ 10^3$ minute 0 1 mg N_2 was taken as a measurement for the activity, four extractions in the glass grinding procedure gave the average value 219 7 for the G 6 P dehydrogenase, and 3 0 for the 6 PG dehydrogenase In the same technique four extractions after sonic disruption of the cells gave the average value 147 0 for the G–6–P dehydrogenase and 8 5 for the 6 PG dehydrogenase

These data are taken to indicate that the glass extraction method is adequate for the release of the G–6 P dehydrogenase In the technique

involving ultrasonic disruption this dehydrogenase is still obtained Among the extra nitrogenous material released, however, is the major part of the 6-PG dehydrogenase The following experiments are concerned with extracts from sonically treated cells

The extracts obtained from sonically treated cells also had some TPNH oxidizing activity This activity had to be taken into consideration in studies of the 6-PG dehydrogenase, since measurements of the latter enzyme activity required fairly large amounts of meningococcal extract The TPNH oxidation was 50–55 per cent inhibited by cyanide or NH_2OH while arsenite, iodacetate and fluoride were without effect Increasing concentrations of TPN reduced the oxidation of TPNH in the way it becomes apparent from some data presented in Table 1

TABLE 1

Effect of the TPN Concentration on the Rates of the 6 PG Dehydrogenation and the TPNH Oxidation in Meningococcal Extracts

TPN μmoles	Rate — A_{340} 10^3 per minute		
	TPNH Oxidation	6-PG Dehydrogenase (Measured)	6-PG Dehydrogenase (Corrected)
None	34	–	–
0 23	33	8	41
1 8	24	83	107
3 6	15	106	121

[illegible] sodium 6-PG or 0 15 μmoles TPNH

The TPNH oxidation in the crude meningococcal extracts resulted in an apparent requirement for unusually high concentration of TPN in order to demonstrate a reaction corresponding to the 6–PG dehydrogenase Some data which illustrate the relation between the TPN concentration and the rates of the 6–PG dehydrogenase and the TPNH oxidation have been recorded in Table 1 The enzyme function corresponding to the 6–PG dehydrogenase appears to give rate substrate concentration curves of the Michaelis-Menton type for TPN such as shown in Figure 1 when appropriate corrections are made for the TPNH oxidation In the figure the data have been arranged according to the method of Lineweaver & Burk A regression line corresponding to the yeast 6 PG has also been included in the figure in order to permit a comparison In these experiments the NH_2OH added also inhibits the Entner-Doudoroff cleavage in the way it has been described below Thus, an oxidation of TPNH formed by a coupling with the Malic enzyme has been eliminated

Naturally, general conclusions should not be based on results ob-

tained with the crude extracts used, in which secondary reactions may give values very different from those obtained with a more purified enzyme. When, with these reservations the data of the meningococcal 6–PG dehydrogenase are compared with those of the crude and the purified yeast enzyme, the K_m of the former with regard to TPN appears to be approximately ten times larger than that of the latter. Such a requirement for high *concentrations of TPN have not been* found in the TPN dependent G–6–P dehydrogenase or isocitric dehydrogenase of N. meningitidis. In the crude meningococcal extracts no significant difference could be found in the activities upon the use of Tris and glycyl-glycine buffers.

The influence of several substances used as inhibitors in various stages of the meningococcal metabolism was also explored. The addition of fluoride, iodacetate, arsenite and KCN were without inhibitory effect in the concentrations generally used, and so was the addition of P_i. Hydrazine apparently increased the dehydrogenation of 6 PG. This effect is assumed to be due, a least partially to an equilibrium change, since the reaction products of the dehydrogenase are trapped by hydrazine. By this trapping, however, ozones are also produced, which due to a very low solubility ,result in a gradually developing turbidity. Thus, during the course of the reaction turbidity to an increasing extent interferes with the spectrophotometric assay. It is as a consequence of these features that hydrazine was not used as trapping agent in experiments which included photometric registration of red ox systems.

Pentose phosphate catabolism. The experiments reported above demonstrate that a synthesis of pentose phosphate from 6–PG may take place in meningococcal extracts. Previous experiments in manometric technique have shown that pentose phosphate is utilized as a substrate in oxidations by meningococcal cells (*Jyssum, Borchgrevink & Jyssum 1961). These features should be elucidated by an investigation* into the routes of pentose phosphate catabolism.

In Fig 2 and Fig 3 some experimental data have been recorded which may agree with the assumption that the pentose phosphates are broken down by a coupling of the enzyme reactions which constitute the conventional pentose phosphate shunt.

From the data presented in Fig 2 it is seen that the decrease in pentose phosphate is accompanied by the accumulation of a compound reacting like seduheptulose in the orcinol reaction, and of GA–3–P. This is in accordance with what might be expected from a transketolase coupled with the enzymes phosphoriboisomerase and epimerase. In this experimental system no synthesis of pyruvic acid takes place, neither has it been possible to demonstrate a synthesis of acetate or acetyl phosphate (*Heath, Hurwitz & Horecker* 1956).

The analyses recorded in Figure 3 furthermore demonstrate that the same reaction system also synthetizes hexose phosphate in the way this may be expected from a coupling of the enzyme reactions men-

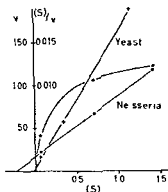


Fig 1

Effect of the TPN concentration on the 6 PG dehydrogenase activities in extracts from N meningitidis and from yeast The meningococcal system is described in the legend of Table 1 The yeast system contained 100 μmoles glycylglycine buffer pH 7 4 and 10 μmoles $MgSO_4$
v = velocity ($\Delta A_{340}\ 10^3$/minute)
(S) = concentration of TPN (mM)
Open circles Plot of v against (S) in the meningococcal system Otherwise (S)/v has been plotted against (S) for an orientation concerning the Michaelis constant

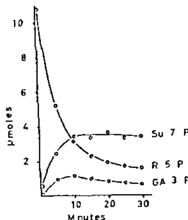


Fig 2

Conversion of pentose phosphate to heptulose phosphate and GA 3 P by cell free extracts from N meningitidis Meningococcus extract dilution 0 4 ml was incubated in a total volume of 1 5 ml with 110 μmoles Tris as buffer pH 7 4 TPP 0 1% μmoles $MgSO_4$ 2 5 μmoles and sodium R 5-P 10 5 μmoles The reaction was stopped at the time indicated by the addition of 1 5 ml 6 per cent $HClO_4$ The supernatants were analysed as described under methods The reaction products did not contain pyruvic acid After incubation for 30 minutes 0 18 μmoles hexose phosphate had been synthetized

tioned above with a transaldolase activity In one of the experimental series in Fig 3 a iodacetate block was included in order to inhibit the GA 3 P dehydrogenase which competes for the intermediate GA 3 P

The Entner Doudoroff cleavage When the quantities of 6–PG which remained unattacked after various periods of incubation with TPN were measured it became apparent that large amounts of the substrate vanished which could not be accounted for in terms of pentose phosphate formation via the 6–PG dehydrogenase No corresponding synthesis of G 6 P could be demonstrated, hence a reduction of the substrate via the G 6–P dehydrogenase could be no factor of importance Obviously meningococci contain some important mechanism which in these experiments favourably competes for the substrate The possibility had accordingly to be investigated of a split of the C–6 compound into smaller units by some reaction which does not include a dehydrogenation Since the Entner Doudoroff type of cleavage appeared to be the most plausible hypothesis attempts were made to demonstrate this pathway

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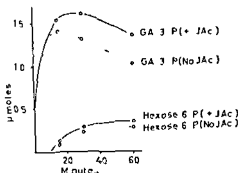


Fig 3

Conversion of pentose phosphate to hexose phosphate and GA-3 P by cell free extracts from N meningitidis

Meningococcus extract dilution 0 5 ml was incubated in a total volume of 2 ml with 130 μmoles Tris as buffer pH 7 4 TPP 0 25 μmoles MgSO₄ 5 μmoles and sodium R 5–P 10 5 μ—

analysed as described under methods No synthesis of pyruvic acid took place in these experiments

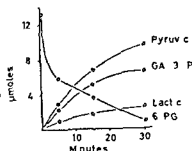


Fig 4

6–PG utilization by extracts from N meningitidis Synthesis of pyruvic acid and GA 3 P

Meningococcus extrat dilution 0 5 ml was incubated in a total volume of 2 5 ml with 105 μmoles Tris as buffer pH 7 4 $CH_2ICOONa$ 20 μmoles K_3AsO_3 20 μmoles and the substrate 13 2 μmoles sodium 6–PG The reaction was stopped at the times indicated by the addition of 2 5 ml 6 per cent $HClO_4$ and analysed as described under methods

sum Borchgrevink, & Jyssum 1961) yields F–6–P, another important substrate for the transaldolase reaction and P_i which is necessary for the GA 3 P dehydrogenase

Since better results might be expected by a trapping of the GA–3 P, an analytical system was also arranged in which the aldehyde was trapped with hydrazine and analysed as alkali labile phosphorus in the technique previously used (*Jyssum Borchgrevink & Jyssum* 1961) Even in this technique however, stoichiometric amounts of the alkali labile phosphorus could not be recovered from 6–PG This is in agreement with the findings of *Wood & Schwerdt* (1954) in their study of extracts from Pseudomonas fluorescens in similar technique It appears likely that the secondary enzyme reactions mentioned above occur at a much higher rate than the reaction with the hydrazine reagent It was found ultimately that a better recovery of GA 3 P could be obtained upon the use of more diluted meningococcal extracts

With the crude meningococcal extracts under study it is assumed that a measure for the activity of the 6–PG splitting enzyme system would be the decrease in substrate quantity, or the quantity of pyruvate formed in the presence of arsenite plus iodacetate

The Entner Doudoroff cleavage in meningococcal extracts was found to be nearly completely inhibited by the addition of KF or NH_2OH, while KCN had no inhibitory effect The experiments performed with

composition of 6-PG by meningococcus extracts results in the synthesis of pyruvic acid, lactic acid and GA-3-P. With the crude extract in question it is obviously *necessary to inhibit* secondary enzyme reactions. *In order* to block a metabolization of the GA-3-P and the pyruvic acid, iodacetate and arsenite were added in the way it has been recorded.

TABLE 2

The Utilization of 6-PG by Extracts from N. meningitidis

Experiment No	Substrate 6-PG µmoles	Inhibitor 20 µmoles of each	Compound measured	Method	Quant. µmoles
1	13.2	None	6-PG	Enz	1.69
			GA-3-P	Enz	6.27
			GA-3-P	Chem	6.10
			Pyruvate	Enz	10.5
			Lactate	Chem	7.40
2	13.2	$CH_2JCOONa$	6-PG	Enz	0.96
			GA-3-P	Enz	6.77
			GA-3-P	Chem	5.30
			Pyruvate	Enz	9.38
			Lactate	Chem	2.60
3	13.2	$CH_2JCOONa$	6-PG	Enz	0.96
		K_3AsO_3	GA-3-P	Enz	6.49
			Pyruvate	Enz	9.63
			Lactate	Chem	2.50

Meningococcus extract dilution 0.5 ml was incubated in a total volume of 2.5 ml with 105 µmoles Tris as buffer pH 7.4 and the substrate. The reaction was stopped after 30 minutes by the addition of 2.5 ml 6 per cent $HClO_4$ and the supernatants were analysed as described under methods.

In Fig. 4 some data have been accumulated which illustrate the fate of the 6-PG when followed in such a system during 30 minutes incubation. It is seen that the reaction results in the synthesis of nearly stoichiometric amounts of pyruvic acid plus lactic acid. The inhibitors added could not completely block the synthesis of lactic acid, presumably via the lactic dehydrogenase (*Jyssum* 1960). The amounts of GA-3-P trapped on the other hand do never reach the quantities expected from an Entner-Doudoroff cleavage. This substance, however, is the common substrate of several reactions which have been demonstrated in meningococcal extracts. Such reactions are, besides the GA-3-P dehydrogenase which is tentatively blocked by iodacetate and a low concentration of P_i, the triose phosphate isomerase and aldolase (*Jyssum, Borchgrevink & Jyssum* 1961), the transketolase and the transaldolase. These enzymes might be expected to transfer the GA-3-P synthetized to a number of intermediates by reactions which cannot easily be blocked. This effect must be assumed to be pronounced since the equilibrium of the aldolase reaction favours the formation of F-1, 6-P. This diphosphate by the effect of the F-1, 6-P phosphatase (*Jys-*

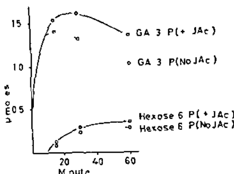


Fig 3

Conversion of pentose phosphate to hexose phosphate and GA 3 P by cell free extracts from N meningitidis

Meningococcus extract dilution 0 5 ml was incubated in a total volume of 2 ml with 130 µmoles Tris as buffer pH 7 4 TPP 0 25 µmoles $MgSO_4$ 5 µmoles and sodium R 5 P 10 5 µm

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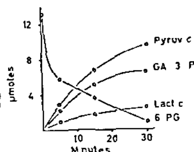


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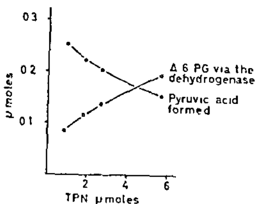


Fig 5

The influence of the TPN concentration on the relative activities of the 6 PG dehydrogenase and of the Entner-Doudoroff cleavage in extracts from N meningitidis Meningococcus extract dilution 0 5 ml was incubated in a total volume of 2 5 ml with 100 μmoles Tris as buffer pH 7 4 $MgSO_4$ 10 μmoles K_3AsO_3 20 μmoles and TPN as indicated in the figure The reaction was started at zero time by the addition of 6 6 μmoles sodium 6 PG and stopped after 10 minutes by the addition of 2 5 ml 6 per cent $HClO_4$ The supernatants were analysed as described under methods The reduction of TPN during the reaction was followed spectrophotometrically and calculations were made in terms of Δ 6 PG Corrections were made for TPNH oxidation

the 6 PG dehydrogenase indicated that the rate of the dehydrogenation could be regulated by means of the TPN concentration as illustrated in Fig 1 It would be of interest to determine the effect of such a regulation of the rate of the dehydrogenation on the activity of the Entner-Doudoroff system In Fig 5 an experimental series has been recorded which demonstrates how the fate of the 6 PG is determined by the available TPN Under the conditions of the assay equal amounts of 6 PG are broken down via the two routes when 4,4 μmoles TPN are added With lower concentrations of TPN the major part is broken down to pyruvate, and with higher concentrations the dehydrogenase appears as the most important catabolic pathway The total quantity of 6 PG which is broken down under these conditions is not significantly different in experiments with 0 2 μmoles TPN, and with 5 μmoles TPN

DISCUSSION

Meningococcal extracts have been found to possess high 6 6 P dehydrogenase activity (*Jyssum Borchgrevink & Jyssum* 1961) The present study is concerned with the further catabolism of the reaction product of this dehydrogenation, the 6 PG

Analyses of the dissimilation of 6 PG show that it is metabolized via two different pathways On the one hand a TPN dependent dehydrogenation to pentose phosphate takes place, and on the other a cleavage into two 3-carbon fragments, presumably according to the system originally discovered by *Entner & Doudoroff* (1952)

The pentose phosphate synthetized by the 6–PG dehydrogenase is apparently broken down in the conventional way by means of a coupling of the enzymes phosphoriboisomerase and epimerase with transketolase and transaldolase.

Maximal activity of the 6–PG dehydrogenase appeared under the conditions of the assay to require higher concentrations of TPN than the yeast enzyme. This difference may be a result of secondary reactions in the meningococcal extracts under study. Several enzyme systems have been considered in this connection.

A coupling with a TPNH oxidizing hydrogenation of 6–PG via the G-6-P dehydrogenase could not be demonstrated. Neither seemed an oxidation of TPNH by the Malic enzyme to be of importance.

The extracts from sonically treated meningococci also had a TPNH oxidizing activity which had to be taken into consideration during studies of the 6-PG dehydrogenase. The effect of cyanide and NH_2OH on the TPNH oxidation may indicate that part of it is the result of a transhydrogenation to DPN, presumably via the glutamic dehydrogenase (*Jyssum & Borchgrevink* 1960). The nature of the TPNH oxidation which was not inhibited by cyanide or NH_2OH has not been explored.

At present the assumption cannot be excluded that the apparent requirement of the 6–PG dehydrogenase of meningococci for higher concentrations of TPN than the yeast enzyme is a real one. In any case the results demonstrate that it may be important to use fairly high concentrations of TPN, probably between 1 and 2 mM, when searching for 6–PG dehydrogenase activity in unknown systems.

In the metabolism of meningococci the 6–PG dehydrogenase *might* be thought unfavourable only to compete for the TPN with other dehydrogenases dependent on lower concentrations of this *cofactor*. Regardless of its K_m in the 6-PG dehydrogenase, however, TPN must be a most important regulator in the activity distribution *between the two* pathways of 6-PG dissimilation in meningococci.

The present experiments have shown that *the 6–PG* dehydrogenase requires treatment with ultrasonic waves in order to be separated from the cellular debris to any significant extent. *This is in* contrast to the G-6-P dehydrogenase previously studied which is released after grinding with glass powder (*Jyssum, Borchgrevink & L*[illegible]

[illegible] particulate matter which is broken up by the ultrasonic treatment into *sufficiently* small pieces to form a stable suspension at the approximately 20 000 g used after the extraction.

SUMMARY

The dissimilation of 6-PG by cell free extracts from N. meningitidis has been explored.

Meningococci possess the enzyme systems necessary for a conversion of 6-PG to pyruvate and GA 3-P corresponding to the Entner-Doudoroff cleavage.

Enzyme catalysed reactions have also been demonstrated which permit a catabolism via the oxidative pentose phosphate pathway.

The influence of the TPN concentration on the activity of the 6-PG dehydrogenase has been studied, and the rôle of TPN as a regulator of the relative activities in the Entner-Doudoroff pathway and the oxidative pentose phosphate route has been discussed.

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A SIMPLE MICROSCALE CENTRIFUGE

By

C G Hedén and E Meyer

Received 13 xii 61

In field conditions, and when it is desired to centrifuge small samples of blood, urine etc, a simple handdriven device may be quite useful For this reason we wish to describe a small unit which has proved to be very useful for the quick separation of serum (for slide agglutination etc) and cells[1]

The centrifuge (cf Fig 1) consists of a round polystyren disc (ø 78 mm, thickness 2 mm) with depressions in the form of grooves on one side The grooves are radial (or placed at a small angle to the radius in order to improve sedimentation) and deep enough to accomodate the centrifuge tubes, which are made of a fine plastic tubing 20 mm long pieces (ø about 1 mm nylon or PVC) are bent in the middle to form a V They are placed in the grooves with the limbs parallel and the joined ends peripheral, before being secured by strips of tape stretched over the grooves

The centre of the disc is symmetrically perforated by two holes 10 mm apart A loop of spun fishing line (terylene, 1 25 m ø 1 mm) is passed through the holes, and the ends knotted on one side

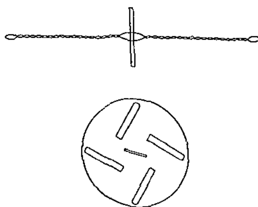


Fig 1

Front and cross section view of rotor disc and string

[1] The apparatus has been used for demonstrations etc by the Sales and PR departments of the de Laval Company Stockholm since 1958

SUMMARY

The dissimilation of 6-PG by cell free extracts from N meningitidis has been explored

Meningococci possess the enzyme systems necessary for a conversion of 6-PG to pyruvate and GA-3-P corresponding to the Entner Doudoroff cleavage

Enzyme catalysed reactions have also been demonstrated which permit a catabolism via the oxidative pentose phosphate pathway

The influence of the TPN concentration on the activity of the 6-PG dehydrogenase has been studied and the role of TPN as a regulator of the relative activities in the Entner Doudoroff pathway and the oxidative pentose phosphate route has been discussed

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A SIMPLE MICROSCALE CENTRIFUGE

By

C G Hedén and E Meyer

Received 13.xii 61

In field conditions, and when it is desired to centrifuge small samples of blood, urine etc, a simple handdriven device may be quite useful For this reason we wish to describe a small unit which has proved to be very useful for the quick separation of serum (for slide agglutination etc) and cells[1]

The centrifuge (cf Fig 1) consists of a round polystyren disc (ø 78 mm, thickness 2 mm) with depressions in the form of grooves on one side The grooves are radial (or placed at a small angle to the radius in order to improve sedimentation) and deep enough to accomodate the centrifuge tubes, which are made of a fine plastic tubing 20 mm long pieces (ø about 1 mm nylon or PVC) are bent in the middle to form a V They are placed in the grooves with the limbs parallel and the joined ends peripheral before being secured by strips of tape stretched over the grooves

The centre of the disc is symmetrically perforated by two holes 10 mm apart A loop of spun fishing line (terylene, 1 25 m ø 1 mm) is passed through the holes, and the ends knotted on one side

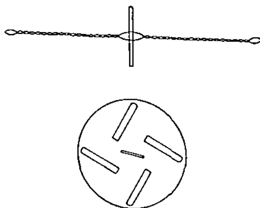


Fig 1
Front and cross section view of rotor disc and string

[1] The apparatus has been used for demonstrations etc by the Sales and PR departments of the de Laval Company Stockholm since 1958

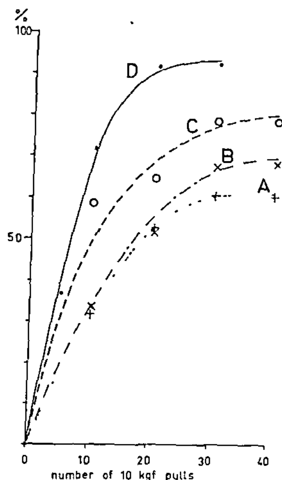


Fig 2

Percentage of the maximum liquid volume (determined in a haematocrit centrifuge) recoverable given as a function of the number of 10 kgf pulls The maximum serum volume obtainable was 57 per cent of the whole blood volume for sample A 62 per cent for sample B and 66 per cent for sample C Sample D consisted of a 10 per cent suspension of yeastcells in saline (1.4×10^9 cells/ml)

OPERATION

Blood is sucked up in a length of tubing which is cut up in a suitable number of pieces These are bent and secured in the grooves The loop on one side of the disc is attached to a hook on the wall, a door handle of some other fixed object A pen or small wooden stick is inserted through the loop on the other side of the disc, which is placed in the middle of the doubled string The pen or stick is now held in one hand, the disc is given a spin with the other, and a process of winding and rewinding is started by rythmically tightening and releasing the pull on the strings This technique, which is well-known to most children gives the disc a very high velocity Of course, the direction of spin is perpetually altered and the tubes are continuously subjected to acceleration and deceleration, but this does not interfere with the sedimentation, probably because of the small tube diameter

After centrifugation the supernatant and sediment are separated simply by cutting the tubing Enough serum can easily be obtained from a piece of tubing to make an agglutination possible

PERFORMANCE

This is best illustrated by a diagram showing an experiment with human citrated blood and a suspension of yeast cells (cf Fig 2) The cell column was measured after every 5–10 pulls (10 kgf) on the string and the results are given as per cent of the maximum liquid volume, i e the volume measured in the haematocrite centrifuge

SUMMARY

A microscale centrifuge consisting of a disc and a piece of fishing line is described It is cheap and easy to operate and should be particularly useful under field conditions

FROM THE UNIVERSITY OF BERGEN, SCHOOL OF MEDICINE
THE GADE INSTITUTE, DEPARTMENT OF MICROBIOLOGY, BERGEN, NORWAY

IMMUNOCHEMICAL STUDIES ON POLYSACCHARIDE A OF STAPHYLOCOCCUS AUREUS

6. *Antigenic Properties*

By

GUNNAR HAUKENES

Received 28.ii.62

A series of publications (6, 7, 8) have dealt with the chemical properties of polysaccharide A. The polysaccharide seemed to be made up of two structures, a ribitol teichoic acid and a mucopeptide. The composition of these structures was found to be very similar to that of the corresponding structures in staphylococcal cells walls. Some investigations have been carried out on the structure of polysaccharide A. These investigations are related to the serological activity of the polysaccharide, for convenience, they shall be presented after this report.

Julianelle & Wieghard (13) found that their polysaccharide A preparation precipitated with sera against virulent staphylococci and not with sera against avirulent strains. They failed to obtain an antibody response to intravenous injections in rabbits (14). *Julianelle & Hartmann* (1936) examined sera from normal individuals and from patients with staphylococcal infections and other diseases. They found that only patients with severe, prolonged or generalized staphylococcal infections produced precipitins to polysaccharide A. All patients with staphylococcal diseases, however, showed cutaneous hypersensitivity to polysaccharide A of the wheal (immediate) type. Like the precipitation, the skin reaction was "type"-specific (*i.e.* group specific, cf. (9)).

In the present study the behaviour of polysaccharide A in the common serological reactions has been examined. Some investigations have been performed regarding the distribution of the polysaccharide in staphylococcal strains, and some attempts have been made to elicit antibodies in rabbits and mice.

MATERIALS

Crude polysaccharide A from strain 1503 prepared as described in (9).
Polysaccharide A Wood 46 purified on DEAE cellulose and Dowex 1 columns originating from the same batch of material as t...
Strains. In addition to 13 coagulase positive st... & *Oeding* (10) 6 strains often used in serological... 680 and 1015 (W. *Kretschmar* Bautzen) strains 5...

ratory Service Colindale London) strain A_4 (*L Grun* Dusseldorf), and strain 830 58 isolated in this laboratory

Two *Staphylococcus aureus* strains commonly used in microbiological research (21) The Oxford strain (Public Health Laboratory Service Colindale London) and strain 209 P (Copenhagen)

J K Schonfeld Rotterdam has furnished us with *Staph aureus* strains which had reverted to the bacterial phase following various numbers of passages in the L" phase The 1365 mother strain and the L_{10} L_{30}, L_{90} L_1 0a L_1 0b L_{019} and L_{301} strains named according to the number of passages before reversion (24)

The *Staph aureus* 18Z mother strain (A) and four mutants B C, D and G were supplied by *F A Kapral* Philadelphia

Four pathogenic *Staph epidermidis* strains were supplied by *I Maclean Smith* Iowa City

Two pathogenic *Staph epidermidis* strains 174 57 (one haemolytic and one non haemolytic apparently variants of the same strain) isolated in this laboratory from a case of septicaemia

Antisera were produced by *Oeding*s technique (19) Antiserum strain Wood 46 and antiserum strain 28 both potent antisera, have been used

Factor *i* serum (18) was prepared by a revised technique Antiserum strain F 21 was absorbed with type strains 3647 and 1503 Strain 3647 has replaced strain 2095 as the latter does not remove all antibodies to the *c* factors present in antiserum to strain F 21

Factor *a* serum was prepared according to *Oeding*s method (18) As mentioned in a previous paper (5) this factor serum is composed of several antibody factors one of which can be absorbed by strain Wood 46 although the strain does not agglutinate Strain 1503 is agglutinated by these agglutinins and serves as the reference strain

All sera used in the serological reactions have been inactivated at 56° C for 30 minutes

METHODS

The techniques used in the *ring test* and in *agar precipitation* have been described in (9)

i

ag

ger

wh

f

c i

pl [illegible] and the complement was thereafter titrated against 2½ units of the amboceptor Two 100 per cent lytic units of the complement and 2½ units of the amboceptor were employed in the present experiments

All procedures were performed in Wassermann tubes using 0 2 ml of each reactant and a final volume of 1 0 ml

The antigen polysaccharide A Wood 46 was *checked for* anticomplementary properties in concentrations up to 0 1 per cent No complement fixation was detected It was also checked that an 0 1 per cent solution of the antigen caused no lysis of sheep cells

In preliminary experiments more complement seemed to be bound in the cold overnight than in two hours at 37° C Moreover zonal inhibition by excess antigen was less pronounced with the former procedure which therefore was preferred It was not determined however to what extent destruction of complement during the binding period might have influenced the results

Sheep cells and amboceptor were mixed and left at room temperature for 5 minutes before use After incubation in a 37° C water bath for 30 minutes the degree of haemolysis was estimated visually

Indirect haemagglutination Sheep cells were washed 6 times in saline and packed in the centrifuge at 1 000 × g for 10 minutes

For sensitization equal volumes of the antigen and 1 per cent sheep cells were mixed and incubated at 37° C for 2 hours The cells were then washed three times and packed as above The supernatant fluid after sensitization was examined by the ring test and by agar precipitation

The test was performed by adding 0.25 ml of a 0.5 per cent suspension of sensitized cells to 0.25 ml of a staphylococcal immune serum which had been absorbed with non-sensitized cells. The contents were incubated at 37° C for 2 hours and left at room temperature overnight before the agglutination was recorded.

The experiments with the crude extract and the crude polysaccharide were carried out by a somewhat different technique (20). Sensitization was achieved with 0.1 ml of packed sheep cells and 1.0 ml of the antigen. The final cell concentration in the haemagglutination test was 1 per cent. Sensitization was attempted also by the use of tannic acid treated sheep cells. The cells were suspended in saline containing tannic acid 1:3,000 and 1:20,000 for 20 minutes in a 37° C water bath and thereafter washed.

All *antibody absorptions* in this work have been performed by incubation in a 37° C water bath for 2 hours with repeated stirring and subsequently left in the ice box overnight before centrifugation.

EXPERIMENTAL AND RESULTS

Precipitation Reactions

The ring test titre of polysaccharide A Wood 46 was examined against various dilutions of antiserum strain Wood 46. A normal human serum was used as a diluent for the antiserum. It appears from Table 1 that the ring test titre remained constant within a wide range of antibody dilutions. Inhibition due to excess antigen is not likely to occur when a potent antiserum is used, as even a 0.8 per cent antigen solution (not included in the table) gave a strong precipitation with undiluted serum. The disc of precipitate formed in the region of excess antigen was positioned below the interface and sank gradually to the bottom of the tube. The ring test titre of polysaccharide A against some other staphylococcal antisera was the same, as might be expected from Table 1.

TABLE 1

Ring Test Precipitation with Polysaccharide A Wood 46 Against Immune Serum Strain Wood 46 Diluted in a Normal Human Serum

Dilutions of 0.1 per cent polysaccharide A Wood 46	Dilutions of antiserum strain Wood 46										
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1,024
1:1	2	2	2	1	1	(1)	(1)				
1:2	2	2	2	2	1	1	(1)				
1:4	2	2	2	2	2	2	2	(1)			–
1:8	2	2	2	2	2	2	2	1			
1:16	2	2	2	2	2	2	2	1	–		
1:32	2	2	2	2	2	2	2	1	(1)		
1:64	2	2	2	2	2	2	2	1	(1)		
1:128	2	2	2	2	2	2	2	1	(1)		
1:256	2	2	2	2	2	2	1	1	(1)	(1)	
1:512	2	2	2	2	2	1	1	1	(1)	(1)	
1:1,024	1	2	2	2	2	1	1	1	(1)	(1)	–
1:2,048	1	1	1	1	1	1	1	1	(1)	(1)	
1:4,096	(1)	(1)	(1)	(1)	(1)	1	1	(1)			
1:8,192	–										

The figures (1), 1, and 2 denote strength of reaction.
– No precipitation in the course of 3 hours at room tempertature.

The characteristic line given by polysaccharide A on agar precipitation has been described previously (9) The approximate amounts of polysaccharide A Wood 46 needed to absorb antibodies to polysaccharide A from two immune sera were examined Aliquots of 50 μl of antiserum strain Wood 46 were absorbed with 4 volumes of the polysaccharide, diluted twofold serially, and examined for free antigen or antibody by agar precipitation Two μg of polysaccharide A was found to absorb all antibodies with excess of antigen Neither free antigen nor antibody could be demonstrated after absorption with 1 μg, while excess antibody was found after absorption with 0 5 μg of the polysaccharide The figures for absorption of antiserum strain 1503 were 0 5, 0 25, and 0 125 μg of polysaccharide A respectively

Complement Fixation

Table 2 shows that the antigen and antibody titres were higher than in the ring test The inhibition of complement fixation in the region of excess antigen is rather pronounced, and shows the importance of using a low antigen dose, *e g* 0 1 μg per ml, when unknown sera are examined for antibodies to polysaccharide A Antiserum strain 1503 gave a titre of 1 64 on progressive dilution of the serum from 1 2, using an antigen dose of 0 1 μg polysaccharide A per ml

TABLE 2

Complement Fixation by Polysaccharide A Wood 46 and Antiserum Strain Wood 46

Dilutions of 0 1 per cent polysaccharide A Wood 46	Dilutions of antiserum strain Wood 46									Saline
	1 5	1 10	1 20	1 40	1 80	1 160	1 320	1 640	1 1 280	
1 20		4	4	2		–			–	–
1 40		4	4	4			–		–	–
1 80		4	4	4	3					
1 160		4	4	4	4		–		–	–
1 320		4	4	4	4	2		–		
1 640		4	4	4	4	4				
1 1 280		4	4	4	4	4	2	–		
1 2 560		4	4	4	4	4	3			–
1 5 120		4	4	4	4	4	4	–		
1 10 240		1	4	4	4	4	4		–	–
1 20 480				1	4	4	4	1		–
1 40 960		–	–		–		3			
1 81 920						–				–
Saline								–	–	

1 to 4 Slight to complete inhibition of haemolysis – Complete haemolysis

Indirect Haemagglutination

Preliminary experiments with a crude polysaccharide A preparation indicated that the polysaccharide was not adsorbed on sheep cells Treatment of the cells with tannic acid was also tried but gave the

same result The crude aqueous extract of disintegrated bacteria of two *Staph aureus* strains, 1503 and 2095, however, sensitized sheep cells The sensitizing component could be precipitated with hydrochloric acid at pH 4 2 and has accordingly been removed on preparation of polysaccharide A The supernatant after absorption of the sensitizing substance by sheep cells was examined by agar precipitation and still produced a strong line midway between the basins This line seemed to be the antigen A (Jensen) line, but no authentic sample of this antigen was available for comparison at that time

Sensitization of sheep cells was also attempted with the purified polysaccharide A Wood 46 preparation using the following amounts 100, 20, 4, 0 8 and 0 16 µg per ml Ring test and agar precipitation of the supernatants showed, however, that no adsorption had occurred The exposed cells were incubated with antiserum strain Wood 46 diluted twofold serially from 1 · 10 to 1 10, 240 No agglutination was recorded

Is Polysaccharide A an Agglutinogen?

Most strains of *Staph aureus* agglutinate at high titres in homologous and heterologous *Staph aureus* antisera (13, 18, 27) Strain Wood 46 was found to be unable to absorb these cross-agglutinins, while most of the other type strains did so, and all of the strains were found to absorb antibodies readily to polysaccharide A Antigen A (Jensen) seemed to be an important cause of the strong cross-agglutination, which, however, might conceal weaker cross-reactions Since strain Wood 46 lacks antigen A (Jensen) and produces a potent antiserum to polysaccharide A (5), this serum seemed to be suitable for examining the rôle of polysaccharide A in the agglutination reactions

One ml of antiserum strains Wood 46 was diluted 1 in 5 in saline and absorbed with 1 mg of polysaccharide A Wood 46 The absorbed serum was found to contain an excess of antigen on agar precipitation Nineteen type strains were agglutinated in the serum using both nutrient agar cultures and mannitol-salt agar cultures (cf under Methods)

Table 3 shows that such agglutination reactions as were abolished by absorption were relatively weak The strong agglutination exhibited by some strains, however, may have masked the agglutinating capacity of polysaccharide A in these strains Cultivation on mannitol-salt agar which disclosed other antigens, did not enhance the agglutinations which may be attributed to polysaccharide A

Factor *i* serum was absorbed with polysaccharide A as above This caused no impairment of the agglutination of the reference strains Γ 21 and Wood 46

Neither did the absorption of factor *a* serum cause any impairment of the agglutination of the reference strain 1503

TABLE 3

Agglutination in Antiserum Strain Wood 46 before and after Absorption with Polysaccharide A Wood 46

Strains	Agglutination titres (reciprocal values)			
	Nutrient agar cultures Antiserum strain Wood 46		Mannitol-salt agar cultures Antiserum strain Wood 46	
	unabsorbed	absorbed with polys. A W. 46	unabsorbed	absorbed with polys. A W. 46
1503	50		50	(10)
2253	50		100	100
28	50		100	100
365	(10)	(10)		–
3647	25	25	10	
F 21	100	100	100	100
17 A	(10)	10	10	
3189	10	–	500	500
2095	(10)		2500	1000
Wood 46	1000	1000	1000	1000
Cowan I	10	–	50	(10)
Cowan II			10	
Cowan III	100		10	
670			100	50
1015	(10)		50	50
830	100	–	2500	2500
5687	10		(10)	–
6376	10		10	–
A_4	10	10	5000	5000

The sera have been diluted 1 : 10, 1 : 25, 1 : 50, 1 : 100, 1 : 250 etc.
( ) Weak agglutination. — No agglutination in 1 : 10 dilution.

It can be concluded that polysaccharide A, under the conditions employed, is a rather weak agglutinogen, and is not the cause of the strong cross agglutination met with in *Staph. aureus* strains. The experiments also showed that polysaccharide A Wood 46 was not contaminated with the ι or the α antigen.

Haemolysis Experiments

As reported under Methods a 0.1 per cent polysaccharide A Wood 46 solution caused no lysis of sheep cells. This was also investigated with rabbit and human cells, but no lysis was observed.

One ml of a standard anti α-toxin serum, containing 20 units per ml, was diluted 1 in 20 and absorbed with 1 *mg* of polysaccharide A Wood 46. The capacity of the serum to neutralize the α toxin was found to be unimpaired.

Thus it appears that polysaccharide A Wood 46 is not contaminated with haemolysins.

The Distribution of Polysaccharide A among Staphylococci

One hundred freshly isolated strains of *Staph. aureus* were examined by agar precipitation against Wood 46 and 28 antisera. When originat-

ing from patients in the same hospital, strains showing the same antibiogram were excluded in order to obtain mainly independent strains. Thick suspensions of the bacteria were heated to 70–75° C for 30 minutes to kill the bacteria, and were then applied to the basins. A 0.01 per cent polysaccharide A solution was added to adjacent basins as a reference for the polysaccharide A line. Ninety-seven of the 100 strains, and all of the type strains except strain 670, produced the specific line.

Staph. aureus strains Oxford and 209-P both gave strong polysaccharide A lines.

Kapral & Li's (15) 18 Z strains all gave polysaccharide A lines.

Of *Schönfeld*'s (24) 1365 strains only the mother strain and the E... strain gave polysaccharide A lines.

Sixty-two strains of *Staph. epidermidis* were examined by the same technique, but no polysaccharide A line was demonstrated. The same was found with *Smith*'s (25) pathogenic *Staph. epidermidis* strains and the two pathogenic *Staph. epidermidis* strains 174/57.

Ten of the 62 *Staph. epidermidis* strains, selected at random, and all of the other strains which produced no polysaccharide A line, were examined for the capacity to absorb antibodies to polysaccharide A. One ml aliquots of antiserum strain Wood 46, diluted 1 in 5, were absorbed with 0.6 g wet living bacteria, *i.e.* the same dose of bacteria as used in the preparation of absorbed sera by *Oeding*'s technique (19) for serological typing. The absorbed sera were examined by agar precipitation against antiserum strain Wood 46 and against a 0.01 per cent solution of polysaccharide A. The results are shown in Table 4.

TABLE 4
Absorption of Antiserum Strain Wood 46 with Staphylococcal Strains which Produced no Polysaccharide A Line

Strains used for absorption	Absorption of antiserum strain Wood 46		
	Excess antibody no. of strains	No free antibody or antigen no. of strains	Excess antigen no. of strains
Ten *Staph. epidermidis* strains	6	1	3
Four pathogenic *Staph. epidermidis* strains (Smith...	4		
Two pathogenic *Staph. epidermidis* strains (174/57)	2		
Schönfeld's 1365 strains			
E70	1		
E90	1		
E170a		1	
E170b		1	
E210	1		
E301		1	
Four *Staph. aureus* strains which gave no polysaccharide A line		4	

Testing for excess antibody or antigen was performed by agar precipitation.

It appears from Table 4 that the 4 *Staph. aureus* strains which produced no specific line were able to absorb antibodies to polysaccharide A. Most remarkably, 3 *Staph. epidermidis* strains produced the polysaccharide A line when this technique was used, and still another strain absorbed the specific antibodies. The line given by the *Staph. epidermidis* strains had the same appearance and showed a reaction of identity with the line of the adjacently placed reference polysaccharide A.

Antibodies to Polysaccharide A in Human Sera

Ten normal human sera with anti-α toxin contents below 0.75 units and 10 patient sera with contents above 3.5 units were selected. The ring test was carried out with undiluted sera against 100, 50, 20, 10, 5, 2 and 1 μg polysaccharide A per ml. Agar precipitation was performed with undiluted sera against 100 and 10 μg polysaccharide A per ml and against a suspension of crushed bacteria of strain 1503. For complement fixation 0.1 μg per ml of polysaccharide A was used as the antigen dose, and the sera were diluted twofold serially from 1:2 to 1:2048.

In no instance could antibodies to polysaccharide A be demonstrated. However, on agar precipitation all sera gave the antigen A (Jensen) line against crushed bacteria of strain 1503, in accordance with the findings of *Jensen* (1958).

Immunization Experiments

Two rabbits were injected intravenously with polysaccharide A Wood 46. The schedule was the same as the one used for immunization with whole bacteria. Three cycles of injections with a 6 day rest period between each cycle. Daily injections for 3 days were given in each cycle. The doses were increased from 5 to 50 μg, and the total amount given was 260 μg. The content of polysaccharide A in the dose of whole bacteria used for immunization, calculated according to the phosphorus content of the cell wall (1), was found to be roughly 200 to 400 μg. Samples taken 7 days after the last injection were examined by the ring test, which was negative.

For immunization polysaccharide A was also prepared in a water-oil emulsion containing killed tubercle bacilli as described by *Freund* (3). Three groups of rabbits, two animals in each, received subcutaneous injections into the interscapular region with the respective doses of 5, 50 and 500 μg of polysaccharide A Wood 46, all in a volume of 1.0 ml. After 4 weeks further injections of 100, 500 and 1000 μg were given to the respective groups. Two groups of mice, 4 animals in each, were injected by the same technique with 0.5 and 5 μg respectively in 0.5 ml, and 5 and 50 μg was given later to the corresponding groups. One rabbit died intercurrently from an unknown cause, and one mouse was killed by another mouse. Blood samples from the ear vein of the rabbits

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Two pathogenic *Staph. epidermidis* strains (174/57)	2		
Schonfeld's 1365 strains			
I 1	1		
I 90	1		
I 1-03		1	
I 1 06		1	
I 51	1		
I 301		1	
Four *Staph. aureus* strains which gave no polysaccharide A line		4	

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Th [illegible]

inj [illegible]

50 [illegible] Wood 46, all in a volume of 1 0 ml After 4 weeks further injections of 100, 500, and 1000 μg were given to the respective groups Two groups of mice, 4 animals in each, were injected by the same technique with 0 5 and 5 μg respectively in 0 5 ml, and 5 and 50 μg was given later to the corresponding groups One rabbit died intercurrently from an unknown cause, and one mouse was killed by another mouse Blood samples, from the ear vein of the rabbits

and the tail vein of the mice, were taken before each injection and 4 weeks after the second injection All samples were examined by the ring test, and the complement fixation test was used also on the rabbit sera as described above All tests were negative

Attempts were made to adsorb polysaccharide A on bentonite for immunization Twenty mg of bentonite (Aluminium silicate, Benton) was added to 1 μg of polysaccharide A in 1 ml of saline The reagents were placed in a 37° C water bath for 1 hour and shaken repeatedly The supernatant after centrifugation gave the same ring test titre as before treatment with bentonite, indicating that no adsorption had occurred

One mg of polysaccharide A Wood 46 was injected intravenously into two mice, and the same amount of the polysaccharide into two rabbits None of the animals displayed toxic symptoms

DISCUSSION

In the ring test, precipitation was obtained within a wide range of antigen concentrations using a potent antiserum This method, therefore, is to be preferred when materials are tested for their content of polysaccharide A Additional testing by agar precipitation, however, is necessary to reveal the nature of the precipitating agent Positive ring tests were obtained also with dilute antisera when a dilute antigen was employed This reaction therefore can be applied also to the testing of sera for antibodies to polysaccharide A, but if so several antigen dilutions should be used Although precipitation was obtained with a relatively high dilution of antiserum strain Wood 46, the reaction was weak and the precipitate did not form a sharp disc Hence, weak and doubtful reactions will always have to be checked by the complement fixation test A highly purified polysaccharide A preparation should be used in these experiments, since weak reactions cannot be checked by agar precipitation Human sera contain precipitating antibodies to antigen A (*Jensen* 1958) Consequently, it is of special importance that the polysaccharide A preparation is free of this antigen

The complement fixation test appeared to be superior to the ring test for estimation of the content of antibodies to polysaccharide A, as it is easily recorded and exhibits a distinct shift from positive to negative reactions The behaviour of polysaccharide A in the complement fixation test agreed with that of pneumococcal polysaccharides The latter also yielded high antigen titres (4), showed zonal inhibition by excess antigen, and bound much more complement in the cold overnight than at 37° C in 1 to 2 hours (22)

At first the distribution of polysaccharide A among staphylococci seemed very clear-cut, as 97 out of 100 *Staph aureus* strains and none of 62 *Staph epidermidis* strains gave the polysaccharide A line Thus this substance seemed to be a group antigen characteristic of *Staph*

aureus and not present in *Staph epidermidis* strains The absorption experiments however complicated the picture as 3 *Staph epidermidis* out of ten now gave the line and a fourth strain absorbed the specific antibody These diverging results must be due to the somewhat different technique used in the latter experiment The use of unheated living bacteria and incubation of the bacteria first at 37° C and subsequent storage in the ice box overnight before they were applied to the basins Consequently two questions arise Is polysaccharide A liberated in greater amounts during growth or released by enzymatical autolysis? These important questions have been examined further and will be reported in the next article

Many other problems are connected with the demonstration of the polysaccharide A line in *Staph epidermidis* strains Is the line caused by an antigen which is identical to polysaccharide A or by one which only is closely related to this polysaccharide? If the antigens are not identical how can they be distinguished and does this related substance occur in *Staph aureus* strains also?

Investigations carried out in this laboratory by *Losnegard & Oeding* (16) have thrown some light on these problems They have shown that the coagulase negative staphylococci cannot be classified as a uniform group by means of one group specific polysaccharide With a modified agar precipitation technique which allowed the bacteria to grow in the basins the polysaccharide A line was produced by a number of *Staph epidermidis* strains which gave no such line when the ordinary technique was used These investigations are in progress and so far no definite conclusions have been reached Some findings however indicate that this antigen is not identical to polysaccharide A (from strains 1503 and Wood 46)

Owing to the complexity of the matter it is at present not possible to draw any conclusions about the specificity of polysaccharide A as a group antigen This problem will also be discussed in the next article in connection with cross reaction experiments with polysaccharide A and staphylococcal wall teichoic acids No investigations have been carried out in this study on possible cross reactions with polysaccharides from other bacterial species

The 18 Z strains are of some interest since one strain 18 Z B lacked bound coagulase (like strain Wood 46) and another strain 18 Z C lacked the soluble type of coagulase Both strains were virulent in mice and rabbits while strain 18 Z D which had both types of coagulase

demonstrated in these strains

resence of the coagulases and

strains in mice and rabbits

Strain 18 Z B did not grow on our mannitol salt agar like other *Staph aureus* strains 18 Z C produced no fibrinolysin and 18 Z G no α toxin (15 17) Accordingly these properties can not also be related to the presence of polysaccharide A

The total lack of polysaccharide A in *Smith*'s 4 strains (25, 26) and the two 171/57 strains indicates that the pathogenicity of *Staph epidermidis* to man is not related to the presence of polysaccharide A in these strains

Schonfeld's 1365 mother strain and the L_{10} strain gave the polysaccharide A line, and only these strains produced phosphatase, hyaluronidase, and fibrinolysin They also, in addition to strain L_{219}, coagulated citrated rabbit plasma, while the other strains coagulated bovine plasma only (23). These strains may be of considerable interest in microbiological and biochemical research, especially with regard to the presence of polysaccharide A in relation to the build-up of the cell wall

None of twenty human sera examined contained precipitating or complement fixating antibodies to polysaccharide A No attempt has been made in this work, however, to collect a comprehensive clinical series for a studying of the important problem of antibody formation in staphylococcal disease

The cause of the negative antibody response in the immunization experiments is not clear It is not likely that it is due to the "immune paralysis" phenomenon observed on immunization with pneumococcal polysaccharides (2), as also very small doses were tried Nor is it probable that the polysaccharide has been deacetylated, since acetyl derivatives of the amino sugars were released on gentle hydrolysis, and as alkalinity was avoided during the extraction and purification procedures It seems more reasonable that the lack of antigenicity is due to a splitting off of the polysaccharide from other high molecular compounds on extraction Some findings have indicated that polysaccharide A is of relatively low molecular weight (6)

With the reservation that other immunization methods might appear to be more successful, our results indicate that polysaccharide A is a hapten

The skin tests carried out by *Julianelle & Hartmann* (12) showed that hypersensitivity to polysaccharide A developed readily *Strominger* (28) has obtained the same skin reaction with a purified sample of the wall teichoic acid from *Staph aureus* strain Copenhagen No skin tests have been carried out in this work

SUMMARY

The behaviour of polysaccharide A in serological reactions has been examined High antigen titres were obtained in the ring test and the complement fixation test It was not possible to adsorb the polysaccharide to sheep red cells or to bentonite

Most *Staph aureus* strains gave the polysaccharide A line on agar precipitation, and the remaining strains were able to absorb antibodies to polysaccharide A Some *Staph epidermidis* strains also produced the

specific line Whether this cross-reaction was due to the presence of polysaccharide A in these strains or to a closely related substance, is not clear

Antibodies to polysaccharide A could not be demonstrated in human sera, and attempts to immunize mice and rabbits were unsuccessful

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IMMUNOCHEMICAL STUDIES ON POLYSACCHARIDE A OF STAPHYLOCOCCUS AUREUS

7. On the Chemical Basis of the Serological Reactivity and the Nature of the Extraction Process

By

GUNNAR HAUKENES

Received 16.1.62

Investigations reported in the preceding paper (10) showed that polysaccharide A, like most bacterial polysaccharides, exhibited haptenic properties. Polysaccharide A was found to be widely distributed among *Staphylococcus aureus* strains, but some *Staph. epidermidis* strains also produced the specific line on agar precipitation.

The present paper concerns cross precipitation experiments with staphylococcal teichoic acid preparations, and some experiments regarding the chemical basis of the serological reactivity. The results of some investigations into the [illegible]
included since they provide [illegible]
saccharide A in the bacteria [illegible]
of the differences in chemical composition between polysaccharide A and the *Staph. aureus* teichoic acids.

MATERIALS

Polysaccharide A Wood 46 prepared as described in (6) [illegible]
sam[illegible]

[illegible]
β [illegible]

One teichoic acid sample from a *Staph. epidermidis* strain, which is a glycerol phosphate polymer with α-linked N-acetylgalactosamine residues (11).

All teichoic acid samples possessed D-alanine ester groups. The samples [illegible] kindly furnished by Professor *J. Bad*[illegible]
of the *Staph. aureus* teichoic acids ha[illegible]

Strains. Strain 1503 (21) and strai[illegible]
m[illegible]nts *Micrococcus lysodeikticus* (N[illegible]

A[illegible]
strain W[illegible]d [illegible]
t[illegible]lysacch[illegible]
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polysaccharide A line and showed a reaction of identity with the latter. After being absorbed with *Staph. aureus* teichoic acid preparations antiserum strain Wood 46 produced no precipitation line against polysaccharide A Wood 46. The amounts of the teichoic acid preparations needed for these absorptions were the same as those found for polysaccharide A Wood 46 (10).

TABLE 1

Ring Test Precipitation with Polysaccharide A and three Teichoic Acid Preparations Against Undiluted Immune Sera

Antisera undiluted	Ring test titres of 0.1 per cent solutions of			
	Polysaccharide A Wood 46	*Staph. aureus* teichoic acid		*Staph. epidermidis* teichoic acid
		Preparation 1	Preparation 2	
Staph. aureus strain 28	1:4096	1:4096	1:4096	Negative 1:1
Staph. epiderm. strain 1254	1:1024	1:16	1:128	1:1024
Staph. epiderm. strain 1268	1:1	1:8	1:64	1:1024
Staph. epiderm. strain 16[illegible]	1:1024	*	*	*

* = weak doubtful reaction without the formation of a sharp disc of precipitate

Electrophoresis

As polysaccharide A and the two *Staph. aureus* teichoic acid samples apparently gave identical ring test titres and agar precipitation patterns it was evident that the mucopeptide moiety was not essential to the serological reactivity. It was of importance therefore to find out whether the mucopeptide was an integral part of the polysaccharide antigen or a contaminant which might be separated from the polysaccharide by electrophoresis.

Polysaccharide A was found to migrate towards the anode both at pH 9.2 and pH 2.37. In N acetic acid pH 2.37 the substance migrated 18 to 20 cm in 3 hours applying a potential of 500 V, i.e. about 17 V/cm. The current was low 3 to 5 mA. In the borate buffer of pH 9.2 polysaccharide A was found to migrate 12 to 14 cm under the same conditions and with a current of 18 to 20 mA.

After electrophoresis the bulk of the serologically active material was contained in a 3 to 5 cm long band. Very small amounts of the material were found between the origin and this band and [illegible]

th ring test tit

METHODS

Paper electrophoresis was performed in N acetic acid or in 0 1M borate buffer pH 9 2 The apparatus was a modification of the one described by *Foster* (4) The material under investigation was applied as a narrow transverse band on a 14 × 40 cm strip of Whatman filter paper No 1 The strip was moistened with the buffer and placed in the apparatus between two glass plates with the *ends dipping into the* reservoirs The power unit was constructed by *Mr Nicolaysen* civil engineer Oslo with equipment for stabilization at a constant potential or at a constant electric current Cooling was achieved by *placing a vessel with ice water on the glass plates* and a fan under the plates The vessel also served to exert a constant pressure upon the upper plate

To reveal the *position of polysaccharide A on the paper after electrophoresis* strips of the paper were cut parallel to the application line and eluted with water for examination by the ring test

Paper chromatography Details of the technique have been given in (8)

Solvent systems A phe … nol saturated with 0 067M ph …) water (6 3 1 v/v), 1 … f v/v) organic phase

Spray reagents 1 0 1 per cent ninhydrin in water saturated n butanol 2 0 4 per cent ninhydrin in n butanol containing 10 per cent phenol 3 sodium periodate benzidine The ninhydrin reagents were acidified with acetic acid

Crushing of the bacteria was carried out in the frozen state in Shandon Hughes bacteria press (13)

Hexosamines were estimated by the method of *Rondle & Morgan* (23)

Enzyme digestion with trypsin and lysozyme was performed by the technique used by *Salton* (24) for cell walls

Trypsin digestion One mg of the material under investigation and 100 µg of crystalline trypsin (Novo) in 1 0 ml of a 0 1M phosphate buffer pH 7 8 were incubated in a 37° C water bath for 2 hours A control without trypsin was included

Lysozyme digestion was performed in 0 1M sodium chloride or 0 1M phosphate buffer pH 6 2 to which was added 0 9 mg of the material and 50 µg of crystalline egg white lysozyme (Sigma) The contents were incubated at 37° C for 24 hours A control without lysozyme was included The efficiency of the lysozyme was assured *by examining its lytic capacity on cells of Micrococcus lysodeikticus* (NCTC 2665)

EXPERIMENTAL AND RESULTS

Cross-Precipitation with Staphylococcal Teichoic Acid Preparations

The ring test titres of polysaccharide A and the teichoic acid preparations were examined against a potent *Staph aureus* antiserum and three *Staph epidermidis antisera* (Table 1)

Polysaccharide A and the *Staph aureus* teichoic acid samples yielded identical ring test titres against the *Staph aureus* immune serum The *Staph epidermidis* teichoic acid preparation, however, reacted with antisera to strains *1254* and *1268* only *Polysaccharide A cross-reacted* more strongly with the *Staph epidermidis* antisera than did the *Staph aureus teichoic acid preparations Antiserum strain 1622* is, according to *Tosnegard & Oeding* (17) a weak antiserum The reactions given by preparations other than polysaccharide A with this serum were weak and doubtful, and the latter showed inhibition in the zone of excess antigen, as a 0 1 per cent solution of the antigen gave a negative ring test

Both *Staph aureus* teichoic acid preparations gave, on agar precipitation, a line of appearance and strength which was identical to the

alanine from the teichoic acid sample To find out whether the whole mucopeptide moiety might have been split off from the rest of the polysaccharide, the hydrolysate was examined by electrophoresis, and subsequently by paper chromatography after acid hydrolysis of the material recovered after electrophoresis Alkali treated polysaccharide A migrated only 4–6 cm towards the anode in N acetic acid, at a potential of 500 V for 2 hours Untreated material migrated 12–14 cm under the same conditions The chromatogram of the acid hydrolysate revealed the same ninhydrin-reacting spots as before, thus the mucopeptide could not be separated from the polysaccharide by this method

Sensitivity of Polysaccharide A to Heat and to Enzymes

Polysaccharide A was found to be heat stable when examined in aqueous solution Autoclaving at 120° C for 2 hours did not influence the ring test titre nor the agar precipitation pattern Heating to 100° C in normal hydrochloric acid and in normal sodium hydroxide, however, led in both instances, to a rapid loss of serological reactivity After 10 minutes' heating 0 1 per cent solutions gave a negative ring test in 1 10 dilution, while the control reacted to 1 4000

Five experiments were carried out to examine the sensitivity of polysaccharide A to trypsin and lysozyme

1 Treatment of polysaccharide A with trypsin
2 Treatment with trypsin as in experiment 1, but employing a polysaccharide A material which had been pre heated to 100° C for 5 minutes
3 In this experiment polysaccharide A was first treated with lysozyme and then with trypsin
4 Lysozyme-treatment of polysaccharide A in 0 1 M sodium chloride
5 Lysozyme treatment in 0 1 M phosphate buffer pH 6 2

All digests were examined by the ring test and agar precipitation The serological reactivity was found to be unimpaired Units of 100 μg of the trypsin digests were examined by a ninhydrin spot test which enabled the detection of 0 1 to 0 2 μg of glycine Units of 450 μg of th

[illegible]

[illegible] treatment of polysaccharide A by heating and with lysozyme was carried out as in accordance with *Salton* (24), trypsin was found to act on *Staph aureus* cell walls pre treated in this way

Extraction Experiments

For the preparation of polysaccharide A crushed bacteria were extracted with water or a slightly acid buffer in the cold The extraction process was investigated in more detail as it seemed reasonable that

The material recovered after electrophoresis was examined by paper chromatography after acid hydrolysis The borate buffer was removed by dialysis and the acetic acid by evaporation Hydrolysis was performed with 6N hydrochloric acid at 105° C for 16 hours After removal of the hydrochloric acid by evaporation, the hydrolysates were run in the phenol-water solvents A, B, and C The papers were treated with ninhydrin (reagent 2) and revealed as before (8) the large glucosamine spot and 5 amino acid spots Some material recovered from electrophoresis in acetic acid, was hydrolyzed with 3N hydrochloric acid for 3 hours at 100° C and run on a chromatogram in solvent system D The paper was sprayed with the sodium periodate benzidine reagents, and the two strongly reacting spots of ribitol and anhydro ribitol were demonstrated (cf (8))

Mild Alkali Hydrolysis

The teichoic acids, obtained by extraction with cold trichloroacetic acid, contain D-alanine connected through an alkali-labile ester linkage (2) Polysaccharide A also contained some alanine, but less than in the teichoic acid samples (9) As a consequence of the unsubstituted amino groups in the alanine, the teichoic acids react strongly with ninhydrin (2) The subsequent experiments were carried out to see whether the alanine linkage in polysaccharide A was labile to alkali and to observe the influence of this treatment on the ring test titre and the mucopeptide linkage in the polysaccharide

Polysaccharide A and the *Staph aureus* teichoic acid, Preparation 2, both in a concentration of 0 1 per cent, were heated to 100° C for 5 minutes in N ammonium hydroxide and then cooled immediately The ammonia was removed by evaporation at reduced pressure

The ring test titres of both hydrolysates against antiserum strain 28 were the same as before, *i e* 1 4 × 10[6] Paper chromatography in the alkaline solvent systems D and E showed that the alanine had been liberated from the teichoic acid preparation but not from polysaccharide A, 100 μg of the latter being applied to the chromatogram After chromatographic separation of alanine the remainder of the teichoic acid at the point of application gave a negative ninhydrin reaction Polysaccharide A, examined in the same way, was ninhydrin negative Thus all, or most, of the alanine in the teichoic acid sample seemed to have been split off

In addition to alanine a weak, more fast moving spot was detected on chromatography of the alkali treated teichoic acid preparation This compound was not found in acid hydrolysates of the material Considering the findings of *Armstrong et al* (2) the new spot seems to represent the amide of the alanine

Accordingly it was apparent that the alanine of polysaccharide A was not released under conditions which caused complete removal of

enzyme might be found in the extract. Crushed, heat inactivated bacteria of strain Wood 46 were suspended in an extract of crushed bacteria of the same strain. As a control crushed, heated bacteria were suspended in another portion of the same extract, which previously had been heated to 100° C for 30 minutes. The ring test titres after 3 days' incubation at 37° C were the same, 1 : 256, in both suspensions, and showed no increase during the incubation. Accordingly the enzyme was not present in detectable amounts in the extract.

TABLE 3

Influence of Pre Heating on the Liberation of Polysaccharide A from Crushed Cells of Strain Wood 46

Crushed cell suspensions heated for 30 minutes at	Ring test titre of subsequent extracts
60° C	1 : 512
70° C	1 : 128
80° C	1 : 32
90° C	1 : 16
100° C	1 : 16
Unheated control	1 : 1024

Suspensions of crushed cells were heated as shown in the table, centrifuged, re-suspended in the buffer and extracted for 24 hours at 37° C. The ring test titre was estimated against antiserum *Staph. aureus* strain 28.

It was reported in the previous paper that 7 strains of staphylococci, although capable of absorbing antibodies to polysaccharide A, did not produce the specific line on agar precipitation. Based upon the present findings attempts were made to demonstrate the line after incubation of suspensions of these bacteria at 37° C for 2 days. However, even with this procedure the specific line was not produced.

DISCUSSION

Polysaccharide A and the *Staph. aureus* teichoic acid preparations showed identical ring test titres and agar precipitation patterns against the *Staph. aureus* antiserum. Since neither the mucopeptide of polysaccharide A, nor the alanine of the teichoic acid is essential for the serological reactivity, this may be due to a common N-acetylglucosaminyl-ribitol grouping or part of it. Cross reaction experiments with teichoic acids from other species, *e.g.* from *Bacillus subtilis*, and with the group A carbohydrate of [illegible]

The three *Staph. aureus* preparations (cf. Table 1) exhibited different reactivity in the *Staph. epidermidis* antisera. Antiserum strain 1622, which reacted with polysaccharide A, contains antibodies to a poly-

the differences found in the composition of polysaccharide A and the teichoic acid from *Staph. aureus* might be explained on this basis. The amounts of polysaccharide A in the extracts in these experiments have been expressed as ring test titres. The identity of polysaccharide A was confirmed by agar precipitation except when quantities were too small, thus making the method inapplicable, i.e. extracts giving a ring test titre below 1 : 16. The extractions were performed with 0.067 M phosphate buffer pH 6.5 and 1 g of wet bacteria suspended in 10 ml buffer.

The yields from crushed and intact bacteria were first compared. Strains 1503 and Wood 46 were extracted for 24 hours in the cold and centrifuged. The bacteria were resuspended and the procedure repeated twice. In the first extract a little more polysaccharide A was obtained from crushed bacteria than from intact bacteria, but after three extractions and on prolonged extraction the yields were found to be identical.

The temperature used for extraction was found to be of great importance as shown in Table 2.

TABLE 2

Influence of Temperature on the Extraction of Polysaccharide A from Crushed Strain Wood 46 Cells

Extraction temperature	Ring test titre of extracts
0° C	1 : 128
4[illegible]° C	1 : [illegible]
37° C	1 : 2048

The extraction was carried out for 48 hours with [illegible] g of crushed cells in 21 ml 0.067M phosphate buffer pH 6.5. The extracts were examined by the ring test against antiserum *Staph. aureus* strain [illegible]8.

In the next experiment suspensions of crushed and intact bacteria of strain Wood 46 were heated to 100° C for 20 minutes and centrifuged. The bacteria were then suspended in the buffer and incubated at 37° C for 24 hours. Suspensions of crushed and intact bacteria treated in the same way except for the heating served as controls. It appeared that heating to 100° C for 20 minutes arrested the extraction process, as extremely small amounts of polysaccharide A could be extracted from the heated bacteria compared with the controls. Very little polysaccharide A material was liberated during the heating period.

The critical temperature for this inactivation was then investigated. The suspensions were heated for 30 minutes in these experiments (Table 3). Polysaccharide A solutions subjected to the same temperatures gave unchanged ring test titres.

The results indicate that the release of polysaccharide A is catalyzed by a heat labile enzyme. It was of some interest to see whether this

The extraction experiments indicate that polysaccharide A is present in an insoluble state in the cell, from which it can be released enzymatically. Ribitol teichoic acids have been found exclusively in the walls of the cells, as far as we know. The obvious presence of this structure in polysaccharide A in connection with another cell wall structure, the mucopeptide, the apparent insoluble state of the polysaccharide in the cell, and the fact that it can be released from intact organisms, lead to the conclusion that polysaccharide A constitutes a part of the cell wall of *Staph. aureus*.

The cell wall lytic enzyme, responsible for the release of polysaccharide A, may be the same enzyme as described by *Mitchell & Moyle* (1957). This enzyme caused lysis of the walls of intact *Staph. aureus* cells with the release of "protoplasts", and could not be extracted from the cells. Another cell wall lytic enzyme of *Staph. aureus* has been described by the above authors and by *Richmond* (22). This enzyme attacked only disintegrated cells and was extractable, accordingly it cannot have been responsible for the liberation of polysaccharide A in our experiments.

Armstrong et al. (2) suggested that the teichoic acid material which was extractable by cold trichloroacetic acid, was associated ionically with other cell wall components. Only part of the wall teichoic acid could be extracted by this method, which indicated that the remainder was bound chemically in the cell wall. *Salton* (1961) showed that extraction with trichloroacetic acid at 100° C removed all teichoic acid mater [illegible] with [illegible] of the [illegible]

Ghuysen (1960) investigated the products released from cell walls of *Bacillus megaterium* by lysozyme, and isolated a substance which shows many parallels to polysaccharide A. The substance was a teichoic acid-mucopeptide complex, the teichoic acid of which being associated with a residue of glucose, N-acetylhexosamines and amino acids. He concluded that this compound represented the wall teichoic acid which was inextractable by cold trichloroacetic acid and linked chemically to the basal mucopeptide of the cell wall, a linkage which cannot be cleaved by cold trichloroacetic acid.

In view of these investigations it is reasonable to consider our polysaccharide A material as a similar teichoic acid-mucopeptide complex.

An interesting parallel exists between polysaccharide A and the group-specific substance of *Streptococcus pyogenes*, the C polysaccharide (*Lancefield* 1928). The latter is, like polysaccharide A, a cell wall component and possesses two sugars, N-acetylglucosamine and rhamnose (*McCarty* 1952). *Krause & McCarty* (1961) compared C polysaccharide materials obtained by different extraction procedures. When extracted by heating the cells to 100° C in 0.05N hydrochloric acid, the polysaccharide was composed only of the two sugars mentioned above. This

saccharide which, if not identical to polysaccharide A, at least shows great resemblance to it (cf (10)) The other two *Staph epidermidis* antisera contain antibodies to another polysaccharide which seems to be widely distributed among coagulase-negative staphylococci (*Losnegard & Oeding* (17)) The specificity of these antisera, however, is not known, and too little is known of the chemical composition of the *Staph epidermidis* polysaccharide antigens to discuss the cause of the observed cross-reactions

Investigations on *Staph epidermidis* polysaccharides are in progress *in this laboratory (17), and* cross-reaction experiments between staphylococcal polysaccharide antigens and various teichoic acid preparations are being carried out in collaboration with *J Baddiley*, Newcastle

Investigations of great interest to this work have been performed by *Juergens et al* (1960) on the chemical basis of the agglutination of cell walls of a *Staph aureus* strain in the homologous immune serum produced by immunization with whole, killed bacteria The agglutination was inhibited by N-acetylglucosamine, but not by N-acetylgalactosamine or N-acetylmuramic acid Moreover, only N-acetylglucosaminides containing *α-glycosidic linkages inhibited the agglutination* The wall teichoic acid of this strain contained α- and β-(N-acetylglucosaminyl)-ribitol residues in a proportion of 1 7, respectively The inhibition by other cell wall components was also investigated The authors conclude that the cell wall agglutination in this strain may be attributed to the presence of α-linked N-acetylglucosamine in the teichoic acid Cell walls of another strain of *Staph aureus*, however, were not agglutinated *by the serum used in the experiments above*

In our experiments, one teichoic acid preparation contained no detectable α-linked residues, but gave the same ring test titre as polysaccharide A and the preparation containing α- and β-glycosidic linkages Most probably, for some reason the above investigators have not obtained antibodies to β linked N acetylglucosamine residues The configuration of the presumed N-acetylglucosaminyl-ribitol linkages of *polysaccharide A has not been established Several polysaccharide A* preparations showed laevorotation (7) In *Staph aureus* teichoic acid this is suggestive of a preponderance of β linkages (3), but the degree to which the mucopeptide components of polysaccharide A may interfere is uncertain

On electrophoresis polysaccharide A migrated towards the anode, even at a pH as low as 2 37, thus exhibiting strong acidic properties The electrophoresis mobility was reduced after mild alkali hydrolysis with ammonium hydroxide, but the ring test titre remained unchanged On further alkali hydrolysis and on acid hydrolysis the serological reactivity was readily lost This was to be expected as both ribitol and N-acetylglucosamine were released on mild acid hydrolysis (8), and alkali hydrolysis will *degrade the polysaccharide*, at least by deacetylation and deamination of the amino sugars

that the N-acetylglucosaminyl-ribitol residues, or part of them, represented the serological active groupings of polysaccharide A

Polysaccharide A was found to be released from the cell walls by a heat labile lytic enzyme It seems reasonable to attribute the different chemical compositions of polysaccharide A and the teichoic acids prepared by extraction with cold trichloroacetic acid, to different extraction procedures

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extraction process removed only a part of the polysaccharide from the cell walls. Formamide extraction removed all of the polysaccharide C material which showed the same composition as above. However, when the cell walls were treated with a cell wall lytic enzyme, the C polysaccharide was released as a mucopolysaccharide, which in addition to the sugars possessed a small peptide moiety. The molar ratios of its amino acids were the same as those of the cell wall amino acids (*Hayashi & Barkulis* 1959).

Baddiley et al. (1961) found that the teichoic acid of *Staph. aureus* H was built up of eight ribitol-phosphate units. The molar composition of polysaccharide A Wood 46 (9) has been estimated to be (Glutamic acid) : lysine : alanine : glycine : N-acetylmuramic acid : N-acetylglucosamine : ribitol phosphate = (0.5) : 1 : 2.1 : 3.0 : 2.7 : 9.5 : 15.7, the ribitol being calculated from the phosphorus value. A structure consisting of 16 ribitol phosphate units and one mucopeptide unit is compatible with the figures above if we disregard the strikingly low glutamic acid value. The molecular weight of this hypothetical structure is 6,500—7,000, too low a value as the hexosamines were not released quantitatively on acid hydrolysis (9). If we reconsider the molar composition of polysaccharide C (12), there seem to be 16 rhamnose units for each peptide unit.

With our present knowledge it is natural, in future work, to prepare polysaccharide A from purified cell walls, preferentially from serological type strains. The immunological significance of the cell wall mucopeptide is unknown, and this problem might be approached by examining the agglutination of purified cell walls by the specific inhibition technique. The crucial question which inevitably arises is: How can the autolysis of the cell walls be prevented without risking degradation of cell wall material? And if the cell wall autolytic enzyme has been inactivated, how then can polysaccharide A and other cell wall components be released? These questions must remain open at present, but in either case it seems to be essential to inactivate the lytic enzyme before purifying the walls and later search for enzymes which act on the purified walls.

Most works concerning the chemical structure of staphylococcal cell walls have been approached from a biochemical point of view. The immunologists, on the other hand, have made little effort to characterize the antigens chemically. It is the author's belief that a wider collaboration in this field of research will prove to be fruitful.

SUMMARY

Polysaccharide A and two teichoic acid preparations from *Staph. aureus* H showed the same serological reactivity with a *Staph. aureus* antiserum. The mucopeptide moiety of polysaccharide A Wood 46 could not be separated from the remainder of the polysaccharide by electrophoresis, and the alanine linkage was not labile to alkali. It was shown

FROM THE CENTRAL BACTERIOLOGICAL LABORATORY OF STOCKHOLM CITY, STOCKHOLM, SWEDEN

THE OCCURRENCE OF CELL WALL CONSTITUENTS IN STABLE PROTEUS L FORMS

By

THOMAS H MORRISON[1] and CLAES WEIBULL

Received 16 iii 62

The occurrence of diaminopimelic acid (DAP) in several stable and unstable Proteus L forms and in pleuropneumonia like organisms (PPLO) was investigated by *Kandler & Zehender* (1957) These workers reported that DAP was present in the unstable L forms but absent in the stable ones and in the PPLO Kandler & Zehender suggested that the absence of DAP in the stable L forms might be caused by a genetic block in the synthesis of DAP

Shortly afterwards *Weibull* (1958) reported that a stable Proteus L form, designated as strain L 9, contained considerable amounts of DAP. Still larger amounts of this acid were found, however, in the normal Proteus mirabilis strain from which strain L 9 was derived Another cell wall constituent, hexosamine, was also found in Proteus L 9 *Weibull & Beckman* (1961) reported that the content of DAP in strain L 9 varied with the age of the culture, more of this compound being found in old cultures than in young ones

In the present paper data are reported on the occurrence of DAP and hexosamine in four strains of stable Proteus L forms

MATERIALS AND METHODS

Cultures

Material used in these studies was four strains of stable L forms derived from Proteus These strains have been carried in penicillin free medium in this laboratory for 6 60 months of continuous passage without signs of reversion to the bacillary form The parent bacillary forms of all four *Proteus* strains were reexamined in this institution and were found to have biochemical properties [illegible]

[illegible]

The authors wish to thank Mrs K *Hammarberg* for [illegible]

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The hydrolysates were spotted on Whatman No 1 filter paper and run as descending one dimensional or two dimensional chromatograms DAP was determined semiquantitatively from the size and intensity of the ninhydrin coloured spot in comparison with controls containing only DAP or a mixture of this acid and other amino acids The precision of the estimations was found to be about ± 30 per cent The solvent for one dimensional chromatograms was the one used by *Weibull* (1958) and the chomatograms were run for about 7 hours The solvent for the first direction in two dimensional chromatograms was the one recommended by *Kandler & Zehender* (1957) i e a mixture of isopropanol water and acetic acid The chromatograms were run for about 20 hours For the second flow the procedure used was that of the one dimensional system

In some experiments the acidic amino acids including cysteic were removed from the hydrolysates by absorption on Dowex 2 (× 8 Ac form) (*Weibull* 1958) The neutral amino acids including DAP were eluted with distilled water

RESULTS

Diaminopimelic acid (DAP) and total hexosamines were estimated in the four strains of Proteus L forms Significantly higher levels of both DAP and hexosamine were found in strain L 9 than in strains L VI, L 18 or L D52

DAP was estimated on paper chromatograms by comparison of the size and colour intensity of the spot with simultaneous controls The limits of detectability of DAP were studied with control chromatograms with mixtures of amino acids and with cell hydrolysates In one dimensional chromatograms DAP could be detected readily in 2 µg quantities and often at 1 µg DAP controls in two dimensional papers could be detected as a trace at 2 µg and could be quantitated at 5 µg

With the chromatographic systems used DAP and cysteic acid move at about the same rate on the papers For this reason, in control experiments the DAP and cysteic acid were separated by neutral and acid elution [illegible]
found howe [illegible]
from cysteic [illegible]
described by [illegible] (1955) When both *pyridine* and hydrochloric acid were present in the chromatogram solvent, the subsequent ninhydrin spot for DAP faded from olive green to lemon yellow within 24 hours Cysteic acid gave a dark purple spot with the ninhydrin reaction and this color faded completely in 24 hours In mixed controls containing twenty times as much cysteic acid as DAP, no trace of the cysteic acid colour was detectable after 24 hours and the DAP spot was indistinguishable from an equal amount of DAP in a homogeneous control For this reason, the Dowex 2 treatment, by which to remove the cysteic acid from the hydrolysates, was usually omitted

Determination of diaminopimelic acid (DAP) in the L form hydrolysates revealed (Table 1) a markedly higher content, on a dry weight basis in cells of L 9 than was found in L VI, L 18 or L D52 Repeated determinations were made on hydrolysates from [illegible]

(*Taubeneck, Böhme & Schuhmann* 1958) and L 18 (*Tulasne* 1949) by Dr *M Sensenbrenner* Strain L D52 was also received from Dr *U Taubeneck* and was originally derived from the Proteus strain 52 described by *Dienes* (1949) The L forms were cultured in the casamino acid broth described by *Abrams* (1955) and grown in the absence of serum and without penicillin or inhibitors Incubation was in flasks at 30° C on a rotary shaker (100 cycles/min) Stock cultures were stored at room temperature and transferred at 2 3 week intervals

Mycoplasma laidlawii strain A was kindly supplied by Dr *E A Freundt* State Serum Institute, Copenhagen It was grown in tryptose broth enriched with 10 per cent horse serum

Crops

Cells used in the chemical analyses were grown in casamino acid broth Flasks were inoculated with about 3 per cent (v/v) of a vigorously growing culture and were ordinarily incubated on the shaker for 48 hours Crops were harvested by centrifugation (Spinco Model L 78 000 × g for 25 min) Resuspension and washing of cells was always performed in supernatant growth medium

Cell crops for the preparation of small body fractions of strain L 9 were usually grown on the casamino acid medium solidified with 0 9 per cent Difco agar (*Weibull & Beckman* 1961 *Weibull & Lundin* 1961) The small body fraction mainly consisting of forms of a diameter < 0 3 µ (*Weibull & Beckman* 1961) was segregated from the larger L elements by differential centrifugation The suspension was first spun at 2 200 × g to remove the bulk of the large (diameter > 0 6 µ) bodies The crude small body preparation was then sedimented from this supernatant by recentrifugation at 78 000 × g The pellets were resuspended in the supernatant growth medium and the cycle repeated The purified small body preparation was obtained by gently resuspending the upper layer of the second pellet Suspensions prepared in this way were examined for homogeneity by phase contrast microscopy If more than 1 large body per 2 fields of view was observed the preparation was repurified by a further cycle of low speed and high speed centrifugation

Determination of Hexosamines

Hexosamines were determined by a modification of the Elson and Morgan reaction using essentially the procedures recommended by *Boas* (1953) and *Gardell* (1958)

A portion of the crop was reserved for dry weight determination and the remainder hydrolyzed in sealed tubes with 4 N HCl for 7 hours at 100° C The acid was then removed by evaporation over the steam bath

Substances interfering with the Elson Morgan reaction for sugar amines were removed by elution from a 5 ml column of Dowex 50 (× 8 H form 200 400 mesh) Aqueous hydrolysate equivalent to 20 100 mg dry weight was introduced into the colum ... lumn to remove chromo
genic ... *Hexosamines* were then
eluted ... ited over the steam bath
and redissolved in water The solution thus obtained containing up to 50 µg of hexosamines was acetylated with fresh acetylacetone reagent at 88 92° for 60 min Colour was formed by Ehrlich s reagent in the presence of ethanol and was read at 530 mµ in comparison to glucosamine standards

Determination of DAP

The DAP content of the organisms was estimated by paper chromatography of hydrolyzed crops The procedures used were derived from those of *Kandler & Zehender* (1957) and *Weibull* (1958)

Since cystine and cysteine are known to give ill defined spots on chromatograms these compounds were converted to cysteic acid by performic acid oxidation prior to hydrolysis (*Dent* 1947 *Thompson* 1954) The oxidation was carried out at room temperature for one hour The preparations were then dried over NaOH in vacuo The residual performic acid was removed by three treatments with distilled water with evaporation to dryness in vacuo

Oxidized cell material was hydrolyzed in sealed tubes with 6 N HCl for 20 hours at 100° C Acid was removed by evaporation to dryness over the steam bath and the hydrolysates were redissolved in water filtered and redried

Analysis of variance revealed that fluctuations among the experiments on the former three strains were insignificant at the 95 per cent level of probability, while the values for L 9 were significantly higher ($P < 0.05$)

In a single experiment the same techniques were used to study the hexosamine content of Mycoplasma laidlawii A The results indicated a hexosamine content of 0 07 per cent This value was probably too high because of interfering colours in the reaction mixtures No interfering colours were ever observed in the L form determinations

The small body fraction of strain L 9 was segregated by differential centrifugation and the diaminopimelic acid content was determined as above Chromatograms were prepared simultaneously from parallel hydrolysates of the corresponding unfractionated crop Four separate agar crops were subjected to this fractionation In three cases, the DAP content of the small bodies (trace—0 1 per cent) was significantly lower than the content of the unfractionated crop (0 3 1 0 per cent) One small body preparation contained insignificantly less DAP than did the corresponding whole crop

In a single preparation, a sufficient yield of small bodies was obtained for determination of hexosamines In this case, total hexosamines constituted 0 48 per cent of the dry weight of the small bodies, while the corresponding whole crop contained 0 74 per cent

DISCUSSION

Detectable amounts of both hexosamine and diaminopimelic acid were found in all four of the stable Proteus L forms studied by us Levels of both substances were consistently significantly higher in strain L 9 than in strains L VI, L 18 and L D52 This is consistent with the differences that may be found among the biological properties of L variants derived from the same bacillary species Furthermore, these strains may have become altered in their growth characteristics by prolonged laboratory passage

The classical descriptive distinction (*Dienes & Weinberger* 1951) between L form growth in the 3 A and in the 3 B phase may be associated with differences in the biochemical composition of the cells According to Dienes the 3 A strains form small colonies on agar, they seldom revert to the bacillary form, and they require serum for growth The 3 B strains form large colonies they revert to the bacillary form on a penicillin free medium and do not require serum for growth *Kandler & Zehender* (1957) describe a third kind of Proteus L form, the C type This L form is similar to the 3 B type except that it does not revert to the bacillary form on penicillin free medium A great number of transfers on a penicillin containing medium is necessary to obtain a stable L form of the C type

According to Taubeneck (personal communication) the Proteus

between 0 1 per cent and 1 0 per cent The corresponding values for the bacillary form of Proteus mirabilis strain 9 were consistently higher in the range of 0 5 per cent to more than 1 0 per cent On the other hand, the DAP in the other three L form strains appeared on the papers as a faint trace and was usually estimated as being no more than 0 05 per cent

TABLE 1

Diaminopimelic Acid (DAP) Content of Stable Proteus L Forms Grown on Liquid Casamino Acid Medium Paper Chromatographic Determinations Values Expressed as Percentages of Dry Weight of Cell Crops

Strain	Diaminopimelic acid	
	Average	Range
L 9	0 3%	0 1 1 0%
L VI	0 05*	trace*
L 18	0 05	trace
L D52	0 05	trace
Bacillary form (Proteus 9)	0 7	0 5 1 0
Mycoplasma laidlawii A	nil#	

* DAP detectable Levels below 0 1% were not quantitated
\# DAP not detectable Levels below 0 05%

TABLE 2

Content of Medium 7 Duplicate

	L form strain			
	L 9	L VI	L 18	L D52
Crop #1	0 78%	0 39	0 [illegible]	0 42
Crop #2	0 93	0 54	0 58	0 42
Crop #3	0 74			
Means	0 82 ± 0 16	0 47	0 42	0 4[illegible]

Mean of L VI L 18 L D52 0 43 ± 0 11
The ± sign indicates 95 per cent confidence limits of mean values

Parallel preparations of Mycoplasma laidlawii A were chromatographed similarly as controls *No trace of DAP could be detected on* these papers The presence of trace amounts of DAP in strains L VI L 18 and L D52 was confirmed by rechromatography after aqueous elution of the appropriate sections of one dimensional chromatograms spotted with up to 50 mg of cell hydrolysate

Hexosamine determinations were made on at least two crops of each strain and each determination was repeated twice The hexosamine content of cells of L VI L 18 and L D52 all fell in the range of 0 4 per cent on a dry weight basis while that of L 9 was about 0 8 per cent (Table 2)

our analyses, since the small body fractions must have been contaminated by fragments of envelopes of large L elements This suggests that the small bodies are structurally different from the large bodies This could be explained if the small bodies were assumed to be formed in the interior of the large bodies and released upon lysis of the latter This is in accordance with light microscope observations (*Dienes & Weinberger* 1951) and the electron microscopic findings of *Thorsson & Weibull* (1958) If formed by such a mechanism, the small bodies would to a large extent be free of the constituents of the large body envelopes

SUMMARY

Diaminopimelic acid (DAP) and hexosamine levels were studied in four stable Proteus L form strains and in the small body (< 0 3 μ) fraction of one strain Paper chromatographic determinations revealed traces of DAP in strains L VI, L 18 and L D52, and about 0 3 per cent in strain L 9 The former three strains also contained significantly less hexosamine than the fourth Less DAP and hexosamine were found in the small body fraction of L 9 than in the whole crops

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strains L VI and L D52 are of the 3 A type. It should be pointed out however that we have been able to grow these strains on serum free medium. According to *Minck, Kirn & Fleck* (1961) the strain L 18 would also be of the 3 A type. On the other hand *Sharp & Dienes* (1959) regard it as a stable variant of the 3 B type. The strain L 9 should be considered as being of the C type since it became stable only after many transfers on penicillin containing medium (*Klieneberger-Nobel* 1956). Thus it is of interest to note that our analyses indicate a chemical similarity between the strains L VI, L 18 and L D52 but a significant difference between these strains and strain L 9.

Considerable evidence has been collected (*Weibull* 1958, *Salton* 1960, *Work* 1961) indicating that DAP and hexosamines are characteristic constituents of bacterial cell walls. *Hoare & Work* (1957) observed small amounts of DAP in soluble fractions of Escherichia coli from which the cell walls had been removed, but in the case of the bacillary form of Proteus 9 *Weibull* (1958) was unable to detect as little as 0.1 per cent in the cytoplasmic fraction. The low levels of DAP found in strains L VI, L 18 and L D52 in our studies, however, cannot be attributed with certainty either to the cytoplasm or to the cell envelope since we estimated the total DAP content of the L forms. Nevertheless it appears probable that the higher levels of this compound and of hexosamine in the L forms of strain L 9 are due to a higher content of cell wall components and that the former three strains either lack such components or contain them at a low level. This hypothesis is in accord with the description of *Taubeneck & Schuhmann* (1959) of the mechanism of formation of the 3 A and 3 B type of Proteus L forms and with the action of bacteriophages on these forms (*Taubeneck, Böhme & Schuhmann* 1958).

Proteus L forms grown in liquid or solid medium consist of individual elements of a spherical shape, the size of which vary within wide limits (*Mandel, Terranova & Sensenbrenner* 1957, *Weibull & Beckman* 1961, *Weibull & Lundin* 1962). Fractions of Proteus L 9 cultures predominantly consisting of elements of a diameter $< 0.3\ \mu$ as measured electron microscopically have been prepared by means of differential centrifugation (*Weibull & Beckman* 1961). These elements do not seem to be able to grow (*Weibull & Lundin* 1962) and have very low biosynthetic activity although they respire vigorously (*Weibull & Beckman* 1961).

If all individual L elements of a Proteus L 9 culture were limited by a cell envelope (cytoplasmic membrane plus modified cell wall) of the same structure, the small bodies might be expected to contain more envelope material than the large ones in terms of per cent of bacterial dry weight. Our analyses indicate rather the opposite. The small body fraction of L 9 contained less DAP and hexosamine than did the unfractionated crops. It should be borne in mind that the small bodies probably contained less DAP and hexosamine than was indicated in

FROM THE SERUM AND TOXOID DEPARTMENT, THE STATISTICAL DEPARTMENT AND THE EPIDEMIOLOGICAL DEPARTMENT, STATENS SERUM INSTITUT, COPENHAGEN, AND THE SCHOOL HEALTH SERVICE, COPENHAGEN

DURATION OF IMMUNITY TO DIPHTHERIA AND TETANUS AFTER ACTIVE IMMUNIZATION

With Mention of Non-Conformity between Haemagglutinating and Neutralizing Diphtheria Antitoxin Titers in some Human Sera

By

I SCHEIBEL, M W BENTZON, S TULINIUS and K BOJLÉN

Received 1 ii 62

An examination of the duration of immunity following diphtheria immunization of children in Denmark published in 1955 (5) showed a satisfactory state of immunity up to 9 years after the last injection

This study has now been followed up on other groups covering a period from 8 to 16 years after the last injection The results are presented in part I of the present article

Part II reports the results of another investigation comprising the duration of immunity to both diptheria and tetanus following immunization with combined diphtheria tetanus vaccine started at an earlier age

PART I

Materials and state of immunity Blood samples were taken from 722 male adults aged 18–28 years originating from all parts of the country

In Denmark immunization of children against diphtheria has been carried out extensively since 1942 and most of the persons included in the study had b[illegible]
immunized in childhood They [illegible]
zation When the blood sampl[illegible]
and if not available they were [illegible]
On the basis of this informatio[illegible]
1 Persons with certificates ve[illegible]
tion 2 Persons who felt conv[illegible] at they had been properly immunized in childhood but who could not produce any certificates 3 Persons who did not know whether or not they had been immunized

A full course [illegible]
each at interval[illegible]
been given The [illegible]
to Al(OH)₃ One [illegible]

Titration met[illegible]
by the haemagg[illegible]
preparation for [illegible]
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mean level of —1 75 viz —1 50 was chosen as the value below which the sera were to be assayed intracutaneously also

TABLE 2

Distribution of Human Diphtheria Antitoxic Sera with I + II Mean Values below and above —1 75 According to their I II Differences *

Differences	$\frac{I+II}{2}$ 1 75		$\frac{I+II}{2}$ >—1 75	
	No	%	No	%
—0 5	2	7 1	20	4 4
0 0	0	0 0	176	38 8
0 5	4	14 3	195	42 9
1 0	7	25 0	57	12 6
1 5	6	21 4	5	1 1
2 0	8	28 6	1	0 2
2 5	1	3 6	0	0 0
Total	28	100 0	454	100 0

* For further explanation see text

Thus the mean values given in the following tables include both the I titers [illegible]

On the assumption that the mean values employed approach very closely the true I mean values the s_I calculated for the 482 sera has been used in the statistical comparison of the results

Results

The mean values of three categories with different records of immunization and of the total number are given in Table 3, together with the number and percentages of persons with less than 0 01 units per ml

TABLE 3

Diphtheria Antitoxin in Adults 12 16 Years after Last Injection

Groups*	No	Units per ml		< 0 01 U ml	
		Log geom mean	Geom mean	No	%
I	235	0 76	0 17	2	0 9
II	324	—0 74	0 18	8	2 5
III	163	0 72	0 19	7	4 3
Total	722	—0 74	0 18	17	2 4

* I Persons with certificate for vaccination
II Persons without certificate but who insist that they were vaccinated in childhood
III Persons who did not know whether or not they had been vaccinated

units per ml but not below or above these levels, whilst *Landy* (13) finds good correlation irrespective of the titers.

However, it became apparent during the work that some of the sera contained diphtheria antitoxin molecules which did not agglutinate the sensitized blood corpuscles although when assayed by the intracutaneous titration in rabbits (11) they appeared to be able to neutralize diphtheria toxin. Furthermore some sera apparently contained a mixture of non-agglutinating and agglutinating neutralizing antitoxin molecules. In no case did haemagglutination occur where no specific antitoxin could be demonstrated.

Thus the haemagglutination titers alone would not give reliable information but rather result in too low an estimate of the immunity level. As it was impracticable to carry out intracutaneous titration of all of the specimens efforts were made to calculate a correction by which the log haemagglutination titers (H) could be related to the log intracutaneous titers (I).

For this purpose 482 of the 722 sera were assayed by the intracutaneous technique irrespective of their H titer. The two sets of titers are correlated in Table 1.

TABLE 1

Relationship between Log Diphtheria Antitoxin Titers in Human Sera Ascertained by Haemagglutination and Intracutaneous Titration

Log haemagglutination titer	Log intracutaneous titer						
	< −2.00	−2.00 to −1.51	−1.50 to −1.01	−1.00 to −0.51	−0.50 to −0.01	≥ 0.00	Total
< −3.00	7	1	8	1	0	0	17
−3.00 to −2.51	1	0	5	0	0	0	6
−2.50 to −2.01	*0*	3	12	4	0	0	19
−2.00 to −1.51	*2*	*11*	48	16	1	1	79
−1.50 to −1.01	0	1	37	70	18	0	126
−1.00 to −0.51	0	0	6	*70*	56	11	143
−0.50 to −0.01	0	0	0	7	*32*	*21*	60
≥ 0.00	0	0	0	0	6	*26*	32
Total	10	16	116	168	113	59	482

Figures in italics indicate corresponding titer intervals.

In 284 sera (59 per cent) the I titer was higher than the H titer, in 176 (36.5 per cent) the titers were uniform and in 22 only (4.5 per cent) the I titer was lower than the H titer.

In order to test whether the relationship of the two titers varied with the antitoxin concentration the sera were divided into two groups, one with I + H mean values below and one with I + H means values above −1.75. This way of expressing the level was chosen in order to give the same weight to both kinds of titers. The I−H differences of the individual sera were calculated and found to vary from −0.5 to 2.5 logarithmic units. Table 2 shows the distribution of the sera within the two groups over this range of differences arranged in steps of 0.5 logarithmic units.

It will be seen that the I−H differences are fairly constant for the 454 sera with I + H values higher than −1.75, 81.8 per cent varying between 0.0 and 0.5. It was therefore justifiable to apply a mean correction for this category. This was found to be 0.25 logarithmic units which should be added to the log H titers in order to give the approximate I titers.

The 28 sera with I + H mean values lower than −1.75 are distributed more evenly over the whole range of differences with a trend towards the higher values, 53.4 per cent varying 1.5 logarithmic units or more against only 1.3 per cent in the other group. To use a mean correction for the lower titers would obviously be misleading. Consequently all sera below a certain H titer were also assayed by the intracutaneous method. To counteract errors in measurements and in individual I−H variations a somewhat higher H titer than the one corresponding to the I + H

exceed the standard error) and thus no decrease in mean titer seems to have occurred from the twelfth to the sixteenth year after the last injection[1]

PART II

Material and state of immunity This part comprises 301 children immunized against diphtheria and tetanus 4 to 8 years previously the great majority of whom started immunization at the age of 5 to 6 months

Immunization was carried out with partly purified combined diphtheria tetanus vaccine adsorbed to $Al(OH)_3$ and containing 50 flocculation units of diphtheria toxoid 12 of tetanus toxoid and 1 mg of Al per ml and was performed along the same lines as described in part I Additional booster doses were not given

Certificates giving exact immunization data were available for all of the children [illegible]

Titration methods The tetanus antitoxin was assayed by subcutaneous injection into mice according to the method described by *Ipsen* (10)

As regards diphtheria antitoxin the same complication as mentioned in part I concerning agglutinating and non agglutinating neutralizing antitoxin molecules was encountered in the sera of the children

Sera from 44 children were assayed intracutaneously irrespective of their H titer and calculations made as described in part I in order to investigate whether the relationship between the two sets of values was alike for adults and children This appeared to be the case and consequently the mean values in the tables in part II have been calculated in the same way as in part I

Concerning the standard deviation this was calculated for both H and I titers for the 44 double tested sera and found to be 0 77 and 0 55 respectively The mutual relationship was the same as for the corresponding adult group However the su[illegible]

Results

The mean values for the diphtheria and tetanus antitoxin and the percentages of children with titers below 0 01 units per ml are given in Table 5

TABLE 5
Diphtheria and Tetanus Antitoxin in Children 5–8 Years after Last Injection

	No	Units per ml		< 0 01 U ml	
		Log geom mean	Geom mean	No	%
Diphtheria	301	—0 89	0 13	11	3 7
Tetanus	301	—0 42	0 38	4	1 3

[1] In testing the significance the standard errors of the differences between the average of the logarithmic titers were estimated by means of the above mentioned standard deviation s_I

It will be seen that the antitoxin levels are almost uniform in the three groups, thus indicating that complete or part vaccination has been carried out on nearly 100 per cent of the age groups involved

Regarding the frequency of titers below 0 01 units per ml, there is *a trend towards* a more frequent occurrence in categories II and III than in category I However, only the difference between III and I is significant (P ≈ 4 per cent) The two persons in group I both possessed some antitoxin – about 0 008 units per ml, one of these was revaccinated and responded with copious antitoxin production Only one of the 15 *persons in the two other groups* showed *traces of* antitoxin, whilst the remaining 14 did not contain measurable amounts These latter were all revaccinated, ten gave a typical secondary response *presenting from* 0 25 to 17 units per ml four weeks later while at that time four had only just measurable amounts, indicating that they might not have been vaccinated previously Actually, in one of these cases this was verified later

About 75 per cent of the well-protected persons were also revaccinated *They all responded with high antitoxin production and showed* about a hundredfold increase in mean titer when assayed four weeks later There was a relatively greater increase among those with lower than higher pre-vaccination titers

The exact knowledge concerning the immunization data for group I made it feasible to examine whether there was any connection between the duration of immunity, age at vaccination, and years after last injection This is shown in Table 4

TABLE 4
Diphtheria Antitoxin in Adults According to Age at First Injection and Years after Last Injection

Age at 1st inj Years	Units per ml geom means Years after last injection				
	12	13	14	15	Total
3			0 39 (2)	0 14 (20)	0 15 (22)
4 5	0 06 (2)	0 16 (25)	0 17 (34)	0 15 (47)	0 16 (108)
6–7	0 26 (10)	0 18 (73)	0 20 (10)	0 25 (6)	0 20 (99)
8	0 36 (4)	0 25 (1)	0 16 (1)		0 35 (6)
Total	0 25 (16)	0 18 (99)	0 18 (47)	0 16 (73)	0 17 (235)

Figures in brackets indicate number of persons

The duration apparently was independent on the age at vaccination in the age-groups investigated, and thus this factor may be disregarded in evaluating the possible *decrease in mean titers in the four years* covered by the study

These mean titers are shown in the last line of Table 4 None of them is significantly different from the total mean (the deviations do not

exceed the standard error) and thus no decrease in mean titer seems to have occurred from the twelfth to the sixteenth year after the last injection[1]

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Tetanus	301	—0 42	0 38	4	1.3

[1] In testing the significance the standard errors of the differences between the average of the logarithmic titers were estimated by means of the above mentioned standard deviation s_I

Four of the 11 children with titers below 0.01 units per ml of diphtheria antitoxin actually contained between 0.008 and 0.004, whilst the remaining 7 had less than 0.005.

Concerning the tetanus immunity, all of the four children with titers below 0.01 units per ml contained some antitoxin, their titers varying from 0.0025 to 0.009.

Table 6 shows an analysis of a possible dependence of age at time of vaccination on the duration.

TABLE 6

Diphtheria and Tetanus Antitoxin in Children 5–8 Years after Last Injection in Relation to Age at First Injection

Age in months at 1st inj	No	U/ml Geom. mean		< 0.01 U/ml			
				Diphtheria		Tetanus	
		Diphtheria	Tetanus	No	%	No	%
2–4	6	0.09	0.29	0	0	0	0
5–6	224	0.12	0.37	11	4.9	4	1.8
7–9	48	0.15	0.47	0	0	0	0
10–16	23	0.21	0.42	0	0	0	0
Total	301	0.13	0.38	11	3.7	4	1.3

It appeared that the 7–9 and 10–16 months groups had significantly *higher mean titers of diphtheria antitoxin than the 2–4 and 5–6 months* groups. The occurrence of the poorly-protected in the 5–6 months groups only may be due to chance.

As regards tetanus, no dependence on age at time of vaccination could be demonstrated, since neither the mean values nor the percentages of poorly-protected in the groups differed significantly.

TABLE 7

Diphtheria and Tetanus Antitoxin in Children in Relation to Years after Last Injection

Years after last inj	No	Units per ml geom. mean	
		Diphtheria	Tetanus
5	26	0.22	1.35
6	109	0.12	0.40
7	163	0.13	0.30

Table 7 shows the fluctuations in the mean values from the fifth to the seventh year after the last injection. The fourth and eight years were represented by only one and two persons respectively and therefore are omitted from the table. In agreement with the results in part I, the diphtheria mean values *do not vary significantly. The decrease*

in the tetanus mean values from the fifth to the sixth year is significant but not from the sixth to the seventh year

The poorly protected children were all found in the sixth and seventh year groups with 5 5 and 1 2 per cent for diphtheria and 1 8 and 1 2 per cent for tetanus respectively This distribution is compatible with chance variation

An analysis of the relationship between duration of immunity age at first injection and period of years after last injection similar to that undertaken in part I Tabel 4 was not considered worth while for the children because of the narrow range of their ages at time of vaccination

DISCUSSION

Diphtheria Immunity

The insignificant fluctuations in the mean values within the two groups during the range of years covered by the present study indicate that an almost permanent level of antitoxin has been established

It is hardly likely that latent infections have played any particular role if any at all in the maintenance of the immunity

Firstly the chances of such infections during the period under observation must have been very small The carrier rate in Copenhagen was found by *Sum* (cit *Bojlen & Scheibel* 5) to have decreased from 13 per cent in March May 1944 to 0 6 per cent in January 1950 No later figures are available but the morbidity has been gradually decreasing and during the last five years no case of diphtheria has occurred in the whole country

Secondly in an earlier study (5) no difference in mean titers was found between a group of children in Copenhagen where diphtheria had been endemic during the years covered by the study and another group from a rural district where no diphtheria had occurred in that period

Similar observations regarding apparently stabilized level of antitoxin after immunization have been made by *Barr & Glenny* (1) in horses and by *Bojlen & Scheibel* (5) in children In this latter study one group of children had a mean of 0 26 units per ml 4 5 years after the last injection and another group had the same mean 8 9 years after the last injection The material comprised 305 children The standard deviation of the logarithmic titers was 0 51 or almost the same as in the present investigation

The significantly lower mean titer for the adults with known date of immunization (part I group 1) 12 16 years after their last injection is compared to the 4 9 years mean for the children in the above mentioned study (5) seems to contradict this hypothesis The two groups were immunized with similar vaccines according to the same schedule and have received the first injection at ages between 1 and 8 years

However the difference in half life of gamma globulin for children and adults may be responsible for the lower level in the adult group

According to *Dixon et al* (7) and *Humphrey & McFarlane* (9), the rates of elimination of homologous and autologous normal or antibody gamma globulin seem to be very similar *Dixon et al* (7) have calculated the half life of these proteins to be approximately 20 days for children and 13 days for adults

To be able to maintain a certain level of antitoxin – A – the rates of production and elimination must be equal The rate of elimination is $\frac{\ln 2}{T}$ or $\frac{0.69}{T}$, T being the half life of antitoxin Under constant production the daily output of antitoxin must therefore be $A \times \frac{0.69}{T}$

To maintain a level of 0 17 units per ml as found for the adults, they must produce $\frac{0.17 \times 0.69}{13} = 0.009$ units per ml per day and to maintain a level of 0 26 units per ml as found for the similarly-treated children (5) they must produce $\frac{0.26 \times 0.69}{20} = 0.009$ units per ml per day The *fact that these two values coincide completely should, of course,* not be overestimated As the children grow older, the half life of their gamma globulin changes from 20 to 13 days Provided the production rate is constant, then the mean titer must decrease gradually to about 0 17 units per ml

The mean for the children in the present study is significantly lower than for the children in the earlier study (5) after the same period of observation The reason for this might be either that the former were immunized with a combined diphtheria-tetanus vaccine, or that *they received their first injection at an earlier age (in most cases when* they were 5 6 months old), or both

However, it is not very probable that the combined vaccine is to blame for the less good effect, since the results of an earlier investigation showed that no anergic effect could be demonstrated in humans immunized with this vaccine (17)

The other possibility seems more likely The diphtheria antitoxin mean values shown in Table 6 are *lower* for children who started immunization when they were 5 6 months old than those vaccinated when they were *7 16 months old* This *indicates* that *maternal* antibodies might have interfered with the response (*i a Barr et al*, 2) The high rate of persons in Denmark immunized against diphtheria and the long duration of the immunity would account for this

It should be noted that no dependence of mean titer on age at time of vaccination could be shown for tetanus antitoxin This agrees well with the slight chance of maternal tetanus antitoxin existing at present, regular tetanus *immunization* only having been carried out since 1950 and in the youngest age-groups only

The *observation* indicates that, under the conditions prevailing in

this country, diphtheria immunization should not be started at too early an age

The daily production of diphtheria antitoxin in this group must be about 0 0046 units per ml in order to maintain a mean level of 0 13 units per ml Provided the rate remains constant, these children will have a mean of about 0 085 units per ml when they reach adult age This corresponds to a 35 per cent reduction The children with a present titer less than about 0 0015 will probably by adult age possess less than 0 01 units per ml 17 such children are included in the group, and thus it might be expected that the frequency of poorly-protected persons in the course of some years will increase from 3 7 to about 5 to 6 per cent

In 1932 *Cl Jensen* (12) presented a method by which to calculate the duration of diphtheria antitoxin immunity, and to our knowledge this is, so far, the only existing attempt in this direction Efforts have been made to compare the results of the present study with that theory *Cl Jensen's* predictions are based on observations during a 2-year period following the maximum titer after a single injection of 150 Lf of diphtheria toxoid Calculations made it probable that the decrease in antitoxin during this period followed the equation $kt = \frac{x}{a(a-x)}$, a being the original amount of antitoxin, x the amount lost at the time, t and k a constant which was found to vary widely from one person to another (0 00084 to 0 354)

The large k variations make it difficult to apply the equation generally In order to overcome this, one of us (*M W Bentzon*) rearranged the equation by introducing $y = a - x$, y being the concentration of the antitoxin present at any time (a = max conc $= y_0$, *i e* y at the time o)

By solving the equation with respect to y, the expression

(1) $$y_t = y_0 \frac{c}{c+t}$$

is obtained where $c = \frac{1}{y_0 k}$.

The reason for doing this is that c varies much less than k from one person to another (from 4 to 121 for the persons in Table 3 (12))

It can be seen from (1) that when t is large in relation to c, the relative decrease in titer will be almost similar for all persons, and that when t is bigger than about 360 days, the rate of disappearance of antitoxin will be almost inversely proportional to time

The results within each of the two groups in the present study do not contradict this assumption, the decrease to be expected during the range of time in question being too small to be registered with certainty However, in the earlier study (5), which covered a wider range of years it should have been possible to register a decrease of the order to be expected As mentioned, this was not the case Also if the results from this study and from the similar-treated adults in the present study are considered together, then, even if the change in elimination be

disregarded, the mean titer of 0.26 units per ml found 4–5 years after the last injection (5) should, according to *Cl Jensen,* have decreased to about 0.08 units per ml 15 years after the last injection. As will be seen from Table 4 this was not the case, the 15 years mean being 0.16 units per ml. It would thus seem that the predictions regarding the duration of immunity based on the calculations of *Cl Jensen* are not compatible with the results observed by us. The same applies to the results of *Fraser & Halpern* (8). When calculating the geometric mean from the figures in their Tabel 1, these are found to be 0.08, 0.06 and 0.07 by the 1st, 2nd and 3rd years after the immunization. The reason for this discrepancy is not known, but it may have some relation to the different methods of immunization.

Tetanus Immunity

The results of the study do not give any information as to whether the significant decrease in mean titer observed from the fifth to the sixth year and the insignificant decrease during the following year indicate a continued decrease at a lower rate or the occurrence of a constant level of a later stage than after diphtheria immunization.

The studies of other investigators in this field covering a period of observation comparable to or longer than ours are all in agreement with our results in showing a long persistence of tetanus antitoxin after immunization (i.a. 3, 4, 14, 15, 18, 22).

However, although it is generally realized that the number of booster doses given influences the level of antitoxin (3, 15, 19, 22), only very few publications give exact data on this point, and there are even fewer in which it is or can be correlated with the time factor.

Sneath & Kerslake (19) after observation of the same 12 persons one and two years after the last of three injections, conclude that practically no decrease had occurred during that time. However, the titers reported do not permit a valid estimation as to whether a 50 per cent decrease has occurred, as could be expected according to *Cl Jensen* (12).

Wishart & Jackson (23) give the geometric means of two groups of about 60 persons who had the same pre booster level of antitoxin 1, 2 and 3 years after a booster dose of either 1 or 10 Lf. The mean values in both groups show some decrease from the first to the second year but none from the second to the third year.

In the study of *Schlegel* (18) covering up to 10 years after the last injection, the same number of injections were administered to all of the persons involved. However, this author gives only the arithmetical means, which makes a comparison within the years rather fictitious, since the individual titers have varied a great deal. In spite of this, the apparant decrease (which most probably is overestimated) is much less than was to be expected according to the theory of *Cl Jensen* (12).

It is not possible at present to give conclusive data regarding a permanent level in the case of tetanus antitoxin but the findings do not conform with the equation of *Cl Jensen* (12) In addition there seems to be no reason for a fundamental difference in this respect between diphtheria and tetanus antitoxin

Provided a constant production rate is valid also for tetanus antitoxin and taking the seven year mean as the constant level the daily output of tetanus antitoxin in the children must be about 0 01 units per ml per day

Later in life a 35 per cent decrease of the mean could be expected and the frequency of unprotected or poorly protected would at that time be expected to be about 2 3 per cent there being at present nine children in the group with titers of 0 015 or less units per ml

CONCLUSIONS

The duration of immunity after active immunization against both diphtheria and tetanus has been found to be very satisfactory using the vaccines and the schedule described

From a public health point of view the state of immunity observed does not in the opinion of the writers at present require the incorporation of regular booster doses into the immunization program as recommended by the health authorities in some countries (*i a* 6)

However it will be necessary to follow up the study at a later date in order to investigate whether or not the expected stability of immunity will materialize

In countries with a high degree of diphtheria immunity in adults immunization of children against diphtheria should not be carried out before the age of about 5 6 months at the earliest

It should be borne in mind that in a certain percentage of human sera the titers of diphtheria antitoxin obtained by the haemagglutination method may be too low as compared with the neutralization titers obtained by the intracutaneous method

SUMMARY

Diphtheria antitoxin has been assayed in the sera of 722 adults most of whom had been vaccinated with $Al(OH)_3$ adsorbed diphtheria vaccine 12 to 16 years previously at the age of 3 to 6 years

Diphtheria and tetanus antitoxin were also assayed in the sera of 301 children all of whom were vaccinated with an $Al(OH)_3$ adsorbed diphtheria tetanus vaccine 5 to 8 years previously when they were about 6 months old

Both the mean levels of antitoxin and the low frequency of poorly protected individuals were found to be highly satisfactory for both diphtheria and tetanus

The results are discussed in relation to the theory of *Cl Jensen* regarding the duration of immunity

Some sera did not give positive haemagglutination although they contained neutralizing antitoxin and some did not haemagglutinate to *full neutralization titer*

For haemagglutinating titers above a certain level a reliable factor for converting the haemagglutinating titer to the neutralizing titer could be computed, whilst this was not possible for titers below this level

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